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# PROCEEDINGS OF THE ROYAL SOCIETY.

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## SECTION B.—BIOLOGICAL SCIENCES.

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### *The Effect on the Blood Sugar of Fish of Various Conditions including Removal of the Principal Islets (Isletectomy).*

By N. A. McCORMICK and J. J. R. MACLEOD, F.R.S.

(Received December 1st, 1924.)

(From the Atlantic Biological Station, St. Andrews, N.B., and the Physiological Laboratory, University of Toronto.)

In fishes the sugar of the blood and the glycogen of the liver have been found to vary considerably even in individuals of the same species, and still more so in those of different species. Practically nothing is known definitely of the causes for these variations, and this we consider an important problem to investigate, especially since light might thereby be thrown on the nature of the metabolism of carbohydrates in cold-blooded animals in which the intermediary stages proceed more slowly than in warm-blooded animals. Our interest was aroused in the behaviour of the blood sugar of fishes for other reasons as well. In certain of the bony fishes (Teleostei) the islets of Langerhans exist as definite glands which have come to be known as the "principal islets." Being more or less separated from the pancreatic tissue itself, these can readily be excised, thus making it possible, by examination of the blood sugar, to determine whether a diabetic condition can be induced by isletectomy without removal of any of the pancreas proper. It was of interest also to see whether insulin can affect the blood sugar. Before such investigations could be undertaken it was necessary to know exactly the degree to which the blood sugar of different fishes of the same species may vary independently of such an operation.

Lang and Macleod (1), in confirmation of earlier work by Diamare (2) and

of Bierry and Fandard (3), found that there are usually only traces of sugar in the blood of the Elasmobranchi, such as *Squalus* (dog-fish), but that considerable amounts may occur in the blood of representative Teleostei, such as *Cyprinus* (carp). In the latter fish it was also noted that the amounts may vary from 0.058 to 0.300 per cent. Fandard and Ranc (4) have stated that the blood sugar in fishes is peculiarly susceptible to asphyxial conditions, but so far as we have been able to find they have published no details of their observations. The most important recent work is that of E. L. Scott (5), who has observed the blood sugar in *Mustelis canis*, the fish prior to the observations being kept in traps which were exposed to tide water and, during them, in shallow tanks. The percentage of oxygen was also frequently determined in the water of the tanks. It was found that no blood sugar, or only traces, could be detected in six out of eight individuals, which are described as having been in a subnormal condition. On the other hand, when the fish were asphyxiated by keeping them out of water for varying periods of time, the blood sugar rose rapidly, to attain, in two specimens, a maximum of about 0.240 per cent. after four minutes, followed by a gradual decline, so that a level of 0.032 was reached in one specimen after 15 minutes. The degree of variability in the results is, however, very great, and they do not seem to us to justify the conclusion that the sugar rises within a few minutes and then falls again during the asphyxial period.

One of us (N. A. McC.) along with E. C. Noble had occasion during the summer of 1923 to observe the blood sugar in numerous sea fishes of various species. These were caught by line and the blood was collected either immediately after landing or some time later, the fish being meanwhile kept in sea-water in a bucket or tub.

The blood was obtained by opening the heart through the gills and allowing it to drop into a crucible with oxalate, and the sugar was determined by the Shaffer-Hartmann method. Typical results are shown in Table I.

The most noteworthy fact demonstrated by these determinations is that a considerable degree of variability occurs, not only when the blood of fishes of different species is compared, but also among individuals of the same species, as in the cases of the sculpin and haddock.

In view of the small range of variability which occurs in the blood sugar of man and other warm-blooded animals under normal conditions, it is difficult to understand why this is not also the case in fishes. To determine the causes for this variability seemed a matter of sufficient importance to investigate more thoroughly, especially since, as already mentioned, it was imperative that

Table I.—Blood Sugar of various Teleostei (mg. per cent.).

Skate ( <i>Raja spec.</i> )	..	..	..	30	38	68	18.		
Witch ( <i>Glyptocephalus cynoglossus</i> )	..			16.					
Sculpin ( <i>Myoxocephalus spec.</i> )	..	..		36	61	34	30	34	(Pail for 30 min.) 10 (fresh) 7.
Haddock ( <i>Melanogrammus æglefinus</i> )	..			36	53	53	28	46	67 64 60 51 60 59 83.
Flounder ( <i>Pseudopleuronectes americanus</i> )				36	60	53	57	51	60 36.
Eel Pout ( <i>Zoarces anguillaris</i> )	..	..		12.					
Sea Raven ( <i>Hemirhamphus americanus</i> )	..			186	8	53.			
Tom Cod ( <i>Microgadus tomcod</i> )	..	..		22.					
Wolf-fish ( <i>Anarhichas lupus</i> )	..	..		14.					
Hake ( <i>Merluccius bilinearis</i> )	..	..		72	65	23.			
Cod ( <i>Gadus callarias</i> )	..	..	..	61	70.				
Herring ( <i>Clupea harengus</i> )	..	..	..	61	58.				

this should be done before attempting to see whether removal of the principal islets might cause hyperglycæmia.

#### *Methods.*

The majority of the observations were made on the sculpin (*Myoxocephalus octodecim spinosus* or *scorpius*) for several reasons. In the first place it is a readily available inshore fish, easy to catch by hand-line, of convenient size and capable of being kept alive for several days in an aquarium, provided the sea water be constantly changed. It is also the only available inshore fish in which the principal islets are sufficiently accessible and isolated, so that they can be excised without any injury to the pancreas.

It was soon observed, however, that the blood sugar in fish taken from the aquarium was, as a rule, decidedly higher than that of freshly-caught fish, even when the water was being frequently changed, so that it became necessary to construct a pen or crib which could be kept in the sea at the depth at which the fish usually occur. For this purpose we constructed a crib, 6 feet square and 3 feet deep, made of a framework of wood (2 × 4 inches) covered by stout netting. By suitable loading with stones the crib was allowed to rest on a ledge of rock, its top, which was hinged to serve as a lid, being 3 feet below the surface of the water at low tide. This precaution was taken so that the crib might not be damaged by wave action, and also so that the temperature of the water might not be influenced on warm sunny days. To transfer fish the

crib was hoisted to the surface and the fish removed by a hand net. After stunning by a blow on the head the pericardial sac was opened and blood removed by a hypodermic syringe, either by inserting the needle into the bulbus arteriosus or by aspirating from the pericardial sac after cutting the bulbus. In the latter case care was taken that no abdominal fluid became mixed with the blood, and frequent comparisons of results obtained on bulbus blood and pericardial blood did not show any difference between the two. From a sculpin of average weight (500 grms.) it is easy to collect from 7 to 10 c.c. of blood directly from the bulbus. Since the blood clots readily, it must be mixed in the syringe with a soluble oxalate. The sugar estimations were made by the Shaffer-Hartmann method and, to control the possibility of dilution, hæmoglobin was measured by the Dare instrument. In all cases the liver was removed and weighed and portions of 5 grms. were usually taken for determination of glycogen by the Pflüger method. In the later observations these portions of liver were preserved in alcohol (95 per cent.) until time could be found to carry through the analyses. It was found by check analyses that this did not interfere with the accuracy of the determinations. The fat was also determined in many of the livers.

### *Results.*

The first observations were made on fish kept in small buckets containing from 3 to 5 litres of sea-water for each specimen. At laboratory temperatures varying between 16° C. and 20° C. the fish under these conditions do not live for longer than 5 hours, and they gradually become very pale from contraction of the chromatophores. Typical results are shown in Table II.

It is clear that the degree of hyperglycæmia which is induced by confinement in stagnant water for similar periods is not the same in different fishes. To determine the factors upon which this variability depends, attention was first of all directed to the glycogen content of the liver, and it will be seen, in the results of July 24, that a parallelism seems to exist between this and the degree of hyperglycæmia induced. Unfortunately glycogen was not determined in most of the other observations of this series, but it was considered that an estimate of its amount might be gained by finding the weight of the liver in relationship to the body weight. Although the amount of fat, as well as the amount of glycogen present in the organ, must affect this ratio, it was thought that the fat would be tolerably constant, so that variability in weight would depend primarily on glycogen. Later results in which glycogen and fat were both determined have not borne out this supposition, so that the liver-

Table II.

Date.	Time in bucket	Weight of fish.	Weight of liver.	Per cent. of liver-weight to body-weight	Per cent. of glycogen in liver.	Blood sugar mg. per 100 c.c.
July 24	Hours	Grms				
	5	255	2.95	1.16	0.007	34
	5	227	5.5	2.44	0.09	126
	5	567	22.2	4.00	1.54	163
	5	312	3.7	1.2	0.006	105
July 23	3½	Large	—	—	5.64	131
	3½	Small	—	—	7.79	168
July 25	3½	255	6.7	2.63	—	92
	3½	397	20.6	5.26	—	181
July 26	2½	757	57	7.70	—	232
	2½	425	—	1.42	—	32
July 27	4½	482	20.6	4.85	—	110
	4½	666	22.45	3.33	—	136
July 28	2½	297	—	—	—	81
	2½	411	12.0	3.00	—	57
August 5	3½	771	59.5	7.7	—	157
	3½	297	25.2	8.3	—	121
August 8	2½ to 3	411	37.6	9.5	—	178
	2½ to 3	482	22.0	4.5	1.12	102
August 9	½	—	16.5	—	—	67
	1	—	2.86	—	—	4
	1½	—	2.91	—	—	126

body-weight ratios are not dependable in estimating the amount of glycogen. It is nevertheless significant that the extent of hyperglycæmia induced by asphyxia often runs parallel with the ratio, as is clearly shown in the results of July 24, 25 and 26 and of August 8. Since such a proportionality did not always exist, however, it was decided that glycogen determinations should be made in all subsequent observations. When we eliminate the experiments in which ratios below 3 occur, it can be seen that the maximum degree of hyperglycæmia is established in about 2½ hours under the conditions of the experiment. The exceptionally low result following asphyxia for one hour in one of the observations of August 9 is difficult to explain, especially since neither the weight of the fish nor the glycogen content of the liver was determined. The very small size of the liver, however, indicates that the organ must have been almost glycogen-free.

In the light of these results it seemed clear that the inequality in the concentrations of blood sugar in line-caught fish recorded by previous investigators, and by McCormick and Noble, was due, in part at least, to variability in the treatment of the fish after catching. Observations were therefore made in which the blood was removed from fish within a minute or two of catching. Typical results are shown in Table III.

Table III.

Date.	How bled.	Weight of fish.	Weight of liver	Per cent. of liver-weight to body-weight.	Glycogen	Blood sugar mg. per cent.	H.B. per cent.
July 25	Pericardium	411	10 2	2.5	Per cent.	48	—
	"	255	3 95	1.6	—	30	—
	"	312	4.3	1.4	—	26	—
	"	354	11 6	3.3	—	24	—
Sept. 10	Pericardium	193	7 8	4.0	—	12	18
	"	208	8.0	3.8	12 0	7	25
	"	177.5	2.5	1 5	6.9	8	45
	"	396	21 0	5 3	8.72	trace	28

There is no relationship between the liver-weight ratio and the blood sugar in these observations. Why the values of July 25 should be so decidedly higher than those of September 10 is impossible to explain, but it is considered probable that these two sets of figures represent the extreme limits between which the blood sugar of normal, fresh-caught sculpin may vary.

*The Rates of Development and of Recovery from Asphyxial Hyperglycæmia.*

As has already been mentioned, E. L. Scott has stated that in dog-fish removed from the water the blood sugar rises rapidly, to reach a maximum in about 4 minutes and then more gradually declines so as to return nearly to the normal level in 6 minutes. These time intervals seemed to us by far too brief, at least for *Myoxocephalus*, so that the observation was repeated. The fish were caught by line and were then left in air for varying periods, after which they were bled. The results are shown in Table IV.

Although glycogen was not determined in all the specimens, this was done in a sufficient number to indicate that the percentage was practically uniform, the fish being caught at rich feeding grounds. No detectable hyperglycæmia occurred until after about 30 minutes, and even up to 45 minutes it was

Table IV.

Time after catching.	Weight of fish.	Ratio of liver-weight to body-weight.	Glycogen in liver.	Blood sugar mg. per 100 c.c.
Min.	Grm.		Per cent.	
9	312	3.93	—	18
17	553	3.07	—	10
23	680	4.66	—	12
24	283	4.48	5.6	32
32	425	5.23	5.8	43
35	539	4.45	7.26	12
38	468	5.89	5.70	24
49	828	2.57	5.66	40
49	539	5.47	—	57
60	397	6.63	—	70
81	595	5.48	5.32	132
98	368	3.38	4.84	96

insignificant, although all the fish did not react similarly. After this time, however, the blood sugar increased rapidly.

In another series of observations the freshly caught fish were kept in the air, wrapped up in moist towels for 20 minutes and then replaced in frequently changing sea-water in tubs. The results are shown in Table V.

Table V.

—	Time after asphyxiating.	Weight of fish.	Ratio of liver-weight to body-weight.	Glycogen in liver.	Blood sugar (mg. per 100 c.c.)	Hæmoglobin.
		Grms		Per cent.		Per cent.*
A Aug. 26	Immediately	269	2.9	5.6	109	26
	1 hr.	212	1.2	—	63	47†
	2 hrs.	241	2.3	0.16	86	34
	3 hrs. 20 mins.	241	1.1	trace	43	32
	4 hrs.	312	2.8	—	262	15
	5 hrs.	255	1.2	—	trace	42
	6 hrs. 40 mins.	340	1.9	3.76	163	—
	11 hrs. 30 mins.	211	2.2	trace	trace	13
	22 hrs.	212	2.1	5.3	135	—
B Aug. 29	1½ mins.	312	5.0	0.11	73	34
	1 min	340	2.4	3.76	91	27
	1 hr. 10 mins.	496	5.0	6.44	164	45
	2 hrs.	354	4.3	10.96	167	28
	4½ hrs.	595	5.2	0.13	135	20
	6 hrs.	425	4.8	8.00	163	20
	7 hrs.	439	3.3	1.76	110	28
	8½ hrs.	439	4.3	2.76	32	35
	9½ hrs.	609	3.4	5.6	59	33
	24 hrs.	482	6.4	6.84	125	29

\* The blood in all these cases was collected through a hypodermic needle inserted in the bulbous arteriosus.

† Blood very scanty.



In both series of observations hyperglycæmia was evident even in the fish taken immediately after the period of asphyxiation, which lasted 20 minutes. This would seem not to correspond with the results given in Table IV, the apparent difference being due to the fact that in this case the intervals are counted from the time of removal of the fish from the sea. When the times elapsing between removal of the fish from the sea (crib) and placing them in the moist towels are taken into account, 20 minutes must be added in the case of A and 25 minutes in that of B of Table V. That the transference of the fish by net from the crib to a tub during which they are exposed to air for 2 to 3 minutes is sufficient to cause some degree of hyperglycæmia is probable, although we have no observations to prove that such is the case.

In its subsequent behaviour the blood sugar attained a maximum in about 2 to 4 hours after the fish had been replaced in frequently changing water. This is most clearly seen in the observations of series B. The results of series A are irregular, no doubt mainly on account of the fact that there was practically no glycogen in the livers of three of the fish\*—namely, in those removed in 2 hours, 3 hours 20 minutes, and 11½ hours respectively. The very low weight ratio in the fish removed in 5 hours indicates that there was little glycogen in the liver. This experiment, perhaps more than any other, shows that a certain percentage of glycogen in the liver is of great importance in determining the behaviour of the blood sugar to such a condition as asphyxia. It is, however, not the only factor, as subsequent observations have shown.

After about 6 hours there is evidence of recovery to the normal level, especially in series B, although this was so slow that considerable hyperglycæmia still remained in 24 hours after the asphyxia. This is of importance in considering the effects of removal of the principal islets.

The hæmoglobin in both the experiments was very high in the fish removed in about one hour after the asphyxia. Its subsequent behaviour in the fish of A was irregular, but in those of B it fell to a subnormal level until the eighth hour, when it recovered to about the normal. For freshly caught *Myoxocephalus* this is about 30 per cent. by the Dare hæmoglobinometer, in which the error of observation is about 5 per cent.

#### *Influence of Frequent Changing of Water and of Temperature on Blood Sugar.*

It has not been found possible to keep the fish in aquaria with frequently changing sea-water so as entirely to prevent the development of hyperglycæmia.

\* The much greater irregularity of glycogen in the fish of A as compared with those of B may be related to the fact that the former had been in the crib for five days and those of B for only two days before removal for these experiments.

The aquaria used consisted either of average-sized tubs or shallow wooden trays, the sea-water in both cases being delivered from piping connected with a water-tower.

In the first three observations, recorded in Table VI, it is seen that in from  $3\frac{1}{2}$  to  $5\frac{1}{2}$  hours in a tub the blood sugar stood at not more than half the height which would be expected in stagnant water. Part of this hyperglycæmia is no doubt to be accounted for by the exposure to air in transferring the fish from the sea. After two days, as seen in the observations of September 11, the hyperglycæmia had subsided considerably. Similar results were obtained by placing the fish in shallow water in the flat aquarium. (Observations of September 1 and 5.) Even under the most favourable laboratory conditions, however, one cannot be certain that the blood sugar will remain at the normal level. After a few days under apparently ideal conditions the fish often become decidedly paler, from contraction of the chromatophores, and when this occurs hyperglycæmia is nearly always present, as in the observation of August 25.

Table VI.

Date.	Time in frequently changing water	Weight of fish	Per cent. of liver-weight to body-weight	Per cent. of glycogen in liver.	Amount of glycogen in liver.	Blood sugar mg. per 100 c c	Hæmo-globin.
		Grms			Grms.		Per cent.
July 23	In tub $3\frac{1}{2}$ hours	638	4 16	4 42	1.175	96	—
	In tub $3\frac{1}{2}$ hours	510	3 85	10 16	2.000	64	—
July 24	In tub $5\frac{1}{2}$ hours	—	—	—	—	77	—
Sept 1	In aquarium several days	255	2 4	—	—	32	—
Sept 5	In aquarium $7\frac{1}{2}$ hours	215	2 55	1 48	0.080	36	18
	In aquarium $7\frac{1}{2}$ hours	204	2.05	1 68	0 71	43	29
Sept. 11	In tub 2 days	248	3 2	—	—	56	29*
	In tub 2 days	532	6 0	—	—	20	32
	In tub 2 days	336	4 8	—	—	20	37
Aug 25	In aquarium 3 days	241	4 7	—	—	316	—

\* Liver curbotic.

*The influence of temperature* on the development of hyperglycæmia in stagnant water was studied early in the investigation before the necessity of considering the glycogen reserves had been demonstrated. Such results as were obtained show clearly that cooling delays the onset of the hyperglycæmia. Thus, in two fish kept in a tub at  $17^{\circ} \cdot 2$  C. for 4 hours the blood sugars rose to 0.116 and 0.274 per cent., whereas in two others kept under similar conditions, except that the temperature was  $9^{\circ} \cdot 5$  C. (by placing the bucket in the ice house), the blood sugars were 0.095 and 0.100 per cent.

In another similar observation the blood sugars in two fish kept at  $17^{\circ}\cdot5$  C. were 0·157 and 0·081 per cent., and in two fish at  $9^{\circ}\cdot7$  C. they were 0·078 and 0·080. A decided retarding influence of cold is evident from these results.

*Behaviour of Blood Sugar of Fish kept in Crib.*

In light of the preceding observations it is to be expected that the hyperglycæmia due to hooking and handling the fish previous to placing them in the crib will take some time to subside. It is important to determine how long this is, not only so that the influence on the blood sugar of various experimental conditions may be determined, but also because the persistence of hyperglycæmia may be related to the subnormal general condition of salt-water fish after returning them to the sea, as in the operation of tagging. The fish are thought by those who have had large experience in this work, which is done for the purpose of studying the problem of migration, to be below par for some time and so to be liable to fall a prey to their enemies.

Fish removed by hand-net were bled at daily intervals after placing them in the crib. In the first observations the blood sugar alone was determined, with the following results :—

Fish placed in pen on July 19—

Removed 2 days later, the blood sugars in two fish were 63 and 73 mg. per cent.

Removed 3 days later, the blood sugars in two fish were 40 and 43 mg. per cent.

Removed 4 days later, the blood sugars in two fish were 99 and 75 mg. per cent.

Removed 5 days later, the blood sugars in two fish were 18 and 24 mg. per cent.

The unexpected persistence of the hyperglycæmia up to the fourth day made it advisable to repeat the observations, especially since no measurement had been made in the above, of the glycogen content of the liver. This is done with the results shown in Table VII.

It is seen, from the third day on, that the blood sugar of the crib fish in 11 out of 13 observations did not exceed that found in freshly caught fish. Occasionally, however, as in one of the fish removed on the fourth and in another removed on the ninth day, somewhat higher values, viz., 53 and 56, were found. These are not associated with an excess of glycogen in the liver and cannot be accounted for in any other way. That they may occur must be borne in mind,

Table VII.

Date.	Days in pen.	Weight of fish.	Per cent of liver-weight to body-weight.	Per cent of glycogen in liver.	Amount of glycogen in liver.	Blood Sugar.	Hæmoglobin.
		Grms.			Grms.	Per cent	Per cent
Aug. 29	1	241	3.3	4.96	0.400	53	30
Sept. 9	1	—	—	—	—	36	30
	1	350	—	4.1	—	205	31
Sept. 10	2	366	4.3	4.7	0.74	20	30
Aug. 30	2	241	3.14	—	—	93	35
Sept. 1	3	297	3.0	3.64	0.33	trace	—
	3	269	5.2	—	—	20	—
Sept. 10	4	213	3.6	3.6	0.28	trace	—
July 28	4	326	3.4	0.39	0.04	30	—
	4	340	4.5	—	—	38	—
	4	638	5.0	—	—	24	—
	4	312	2.4	—	—	53	—
Sept. 12	4	162.5	1.5	—	—	trace	—
	4	368	4.2	—	—	32	—
Aug. 6	6	283	7.4	—	—	36	—
	6	255	7.4	—	—	24	—
Aug. 9	9	—	—	—	—	56	—
Sept. 9	11	—	—	2.08	0.20	24	30

especially in investigating the effect of experimental conditions, such as isletectomy, on the blood sugar.

When a *prolonged period of asphyxia* (20 minutes in air) precedes placing the fish in the crib the hyperglycæmia may persist for much longer than when this is only of short duration, such as was the case in the observation of Table VII. To serve as controls for observations in which the islets were removed, it was necessary to ascertain for how long the hyperglycæmia would last in fish exposed to the air for the same time as the operation of isletectomy. The results are shown in Table VIII and also charted in the diagram on p. 20.

These observations show that the hyperglycæmia may persist even until five days after the asphyxiation (Nos. 287 and 158). After this period, however, none of the blood sugars went beyond the upper limit of 35 mg. per cent. observed in fresh-caught fish. The marked hyperglycæmia seen in fish No. 287 is impossible to account for, there being practically no glycogen in the liver. It is noteworthy that there was also unusually little fat and that the hæmoglobin was only 18 per cent.

#### *The Relationship of Glycogen to Asphyxial Hyperglycæmia*

Although it is clear that asphyxia, due either to removal of the fish from water or to inadequate replacement of the latter, is a potent cause of hyper-

Table VIII.—Effect of 15 to 20 minutes asphyxia on subsequent behaviour of Blood Sugar.

No.	Date of asphyxiation	Days after asphyxiation	Blood sugar per cent	Hæmoglobin	Fish weight.	Per cent of liver-weight to body-weight.	Glycogen in liver	Fat in liver.	Remarks.
201	August 19	1	Mgs. 201	Per cent. 30	Grms. 312	2.3	Per cent. 2.3	—	Asphyxiated about 20 m. by placing in towel.
132	" 30	2	0.045	—	—	—	—	—	
271	" 20	3	127	—	425	2.7	4.24	—	
284	" 19	4	53	33	382.5	3.7	1.60	—	
287	" 20	5	367	18	226	2.0	—	14.3	
158	" 30	5	67	—	348	2.3	1.36	—	
166	September 4	9	trace	—	204	2.1	3.3	5.68	
197	August 30	10	34	28	235	2.2	0.24	—	
136	" 30	10	32	30	250	2.2	6.36	26.8	
168	September 4	10	trace	27	231	2.5	0.09	12.8	
148	" 4	10	36	30	408	3.1	0.88	—	
142	August 30	13	28	16	279	3.1	0.054	27.6	
276	" 20	24	20	—	—	—	—	32.7	

glycæmia, there are evidently certain other factors which may have the same effect or which may greatly influence that of asphyxia. We thought at first that these might be dependent upon whether or not the fish had recently been feeding, and to put this possibility to the test we divided the crib into two compartments by netting and placed large amounts of food in one of them. It was found unreliable to determine the possible influence of feeding in this way, mainly because only certain of the fish in the "fed" compartment would take the food. There was also the risk that certain of the supposedly starved fish would get some of the food by its being washed by tide currents through the meshes of the dividing net. It was partly to control this factor that it was decided to determine the glycogen content of the livers. A less laborious method would have been to examine the stomach contents, but it was found in the cases in which this was done that not infrequently the stomach was empty when it seemed certain that food had been taken within a day or so. It was mainly for this reason that it was considered that a much safer criterion of the nutritive condition of the fish would be the glycogen content of the liver.

The relationship between the degree of asphyxial hyperglycæmia and the liver glycogen has already been referred to in connection with the results in Table II. It is also seen very distinctly in Table V, in which asphyxia failed to cause any hyperglycæmia in two fish in the livers of which no glycogen could be detected and only a very slight degree of hyperglycæmia in another. But there are evidently wide limits between which the amount of glycogen in the liver does not influence the behaviour of the blood sugar following asphyxia. Thus, when the total amount of the glycogen exceeds 0.05 gm. there does not appear to be any relationship. So long as the glycogen stands at a certain fairly low level, its mobilization as sugar in response to the establishment of an asphyxial condition is maximal, and the only influence which it probably has is on the duration of the hyperglycæmia. Since this might subside for another reason, however (namely, the disappearance of the asphyxial condition itself), it is impossible to put the suggestion to the test. The ratio of liver-weight to body-weight has not proved to be of much value as a guide either to the amount of glycogen or the sensitiveness of the blood sugar to change. It does, however, indicate in a rough way whether food has recently been assimilated, and it will be seen, as in the observations following asphyxia (Table VIII), that it is usually below 3 in fish that have been kept in the crib for any length of time.

The failure to demonstrate any close relationship between the extent and

duration of asphyxial hyperglycæmia and the glycogen content of the liver raises the question whether the extra sugar which appears in the blood in such conditions may be derived, in part at least, from "masked" carbohydrates in the blood itself, such, for example, as gluco-proteins, lower polysaccharides, or substances of a glucosidal nature. To investigate this possibility we have measured the change in reducing power caused by heating the blood in the presence of various concentrations of mineral acid (HCl). The following are typical results.—

I. *July 12*.—

- (1) Mixed blood from 5 fish contained 0·024 per cent. glucose.
- (2) Protein-free filtrate heated on boiling water bath with 1 drop HCl (conc.) contained 0·036 per cent. glucose.
- (3) Blood heated in presence of 2 per cent. HCl contained 0·075 per cent. glucose.

II. *July 14*.—

	I.	II.	III.
Fresh blood from each of 3 fish kept some time in tub contained . . . . .	0·059	0·058	0·191 per cent. glucose.
Same blood in each case but after heating for 1 hour in presence of 0·1 NHCl contained	0·101	0·099	0·199 „ „

III. In the following cases the filtrates by the Shaffer-Hartmann method were hydrolysed in the presence of 2 per cent. HCl:—

*Mg. Glucose per 100 c.c. Blood.*

	Before hydrolysis.	After hydrolysis.
Skate . . . . .	30	40
Sculpin . . . . .	34	22
	34	42
	61	104
Haddock . . . . .	46	65
	83	86
Flounder . . . . .	36	51
	57	64
Sea Raven . . . . .	53	60
Wolf-fish . . . . .	14	36
Cod . . . . .	70	105

It is evident that a considerable amount of masked carbohydrate occurs in fish blood, as evidenced by the increase in reducing power which can be caused by hydrolysis. Generally, this is much more marked when the blood itself is hydrolysed than when the protein-free filtrate as obtained in the Shaffer-Hartmann method is employed. This would seem to indicate that the masked carbohydrate exists in some colloidal form which is precipitated along with the proteins, and to judge from the reducing power after hydrolysis it is possible, in sculpin blood at least, to account for most of the sugar which appears as a result of asphyxia as coming from it. It was not possible to carry these investigations to completion, but it may be said that we have obtained other evidence which supports the view that the "sugar" in the blood of fishes is in certain particulars different from that of mammals.

Observations were also made on the process of *glycolysis*. It has been found that this does not occur within several hours in the oxalated blood of the sculpin kept at room temperature. Thus —

Blood removed (on August 29) at 3 : 05 from an asphyxiated fish contained 0.135 per cent. sugar. After standing 6½ hours, the test-tube being meanwhile frequently shaken, it contained 0.131 per cent.

Blood from another asphyxiated fish removed at 11 : 38 contained 0.164 per cent sugar. After 10 hours without shaking it contained 0.167 per cent.

Mixed blood removed from several freshly caught fish contained 0.024 per cent sugar. After standing 1 hour 10 minutes it contained 0.024 per cent. After 3 hours 10 minutes 0.024, and after 21 hours 0.036 per cent.

It is, of course, possible that the oxalate may have interfered with the glycolytic process, since it has been found by one of us to have this effect on glycolysis in mammalian blood (11).

Having determined, as far as was possible in the time available, the conditions under which changes occur in the blood sugar when the fish are kept for the same time and handled to the same extent as would be necessary in investigating the influence on it of various experimental conditions, the effects of injections of epinephrin and insulin and of removal of the principal islets were investigated.

#### *The Effect of Epinephrin.*

There is no more certain way for producing hyperglycæmia in mammals than by the subcutaneous injection of epinephrin (adrenalin chloride). It was therefore considered important to see whether the same is true in fish.

Thirty minutes after removal from the crib, 1 c.c. adrenalin chloride was injected into each of four fish, which were then kept in running water. Two



fish were placed under the same conditions, but were not given epinephrin. The results are shown in Table IX.

Table IX.

Time after epinephrin.	Weight of fish.	Per cent of liver-weight to body-weight.	Per cent of glycogen in liver.	Glycogen of liver.	Blood sugar.	Hæmoglobin.
	Grms			Grms.	Per cent.	
55 mins	324	4.4	11.2	1.60	101	28
1 hr 45 mins	240	2.08	0.5	0.025	173	—
4 hrs 15 mins	255	—	1.3	—	188	—
7 hrs 15 mins	412	2.9	0.1	trace	88	28
Control 1	215	2.5	1.48	0.082	36	18
Control 2	204	2.05	1.68	0.071	43	29

The blood sugar rose rapidly, gaining a maximum in 1 to 4 hours and then declining. Shortly after the injections the fish became excessively pale from contraction of the chromatophores, and since there could be no doubt that the blood sugar responds in the same way as in mammals, the experiment was not repeated.

#### *The Effect of Insulin.*

At an early stage in the investigation of the effect of insulin on the blood sugar of normal warm-blooded animals a few observations were made by us to ascertain its action on cold-blooded animals. Insulin was injected into the dorsal lymph sac of frogs, and these were kept for several days at average room temperature. Even when relatively massive doses were injected the frogs were not observed to develop any symptoms, although they were kept in the laboratory until the fourth day following the injection. A few months later A. Krogh informed us that he had found that when the frogs were kept after injection for longer periods of time symptoms supervened which were comparable with those induced by insulin in mammals. The frogs became hyperexcitable, unable to maintain their equilibrium, and they often showed convulsive seizures, the symptoms being relieved by glucose. A repetition of the observations in this laboratory in the spring of 1923 by J. M. D. Olmsted confirmed these findings, and it was further observed that the onset of the symptoms could be accelerated by warming the injected frogs. Attempts to determine whether the blood sugar became gradually lowered preceding the onset of the symptoms failed, because of the fact that in frogs kept for some time under laboratory

conditions this is very low (about 20 mg. per 100 c.c.). Having noted that the blood sugar rises considerably in frogs when they are kept for two days at 28° C., Olmsted found that insulin does not cause a lowering of this abnormally high blood sugar. Observations on the effect of insulin on frogs were made later by Julian Huxley and Fulton (7), who found that a period lasting several days supervened between the injection and the appearance of symptoms. This could be shortened by warming the frogs; thus it took from five to six days for symptoms to appear when the animals were kept at 7° C, whereas it took only 24-27 hours in the case of those kept at 25° C. Two other important facts were also noted, namely, that the time of incidence of symptoms bore no relationship to the dose of insulin within wide limits, and that frogs kept cool for some days after injection quickly developed symptoms when subsequently warmed. In the summer months of 1923 Olmsted continued his studies on the effect of insulin on cold-blooded animals, using fresh-water catfish (*Ameiurus nebulosus*) and described the development of peculiar symptoms supervening in about two days following the injections, the fish being meanwhile kept at room temperature. These could be only temporarily relieved by injections of glucose. One of us (N. A. McC.) working at St. Andrews also observed peculiar symptoms to develop in the Sculpin (*Myoxocephalus*) and Sea Raven (*Hemirhamphus*) several days after the injection of insulin.

Assuming that the symptoms observed in these cases are related to a fall in blood sugar to a certain low level, as is the case in mammals, it would appear that insulin causes hypoglycæmia in cold-blooded animals just as it does in mammals. However, this is not inevitably the case, and as a matter of fact Noble and Macleod (8) have been unable to demonstrate any fall in blood sugar as a result of the administration of insulin to turtles. Houssay, Sorddelli and Mazzacco (9) and later Houssay and Rietti (10) have also published brief statements of investigations of the effects of insulin on different cold-blooded animals, including frogs, toads, turtles, snakes and fish. In the latter no special symptoms were observed to follow the injection of large amounts of insulin.

Apparently, therefore, nothing definite is known concerning the effect of insulin on the blood sugar in fish, and it was decided to study this question in sculpin, with the results shown in Table X.

Since the blood sugar in the normal sculpin is not infrequently practically at zero it is not possible to demonstrate any effect of insulin on it. This was attempted in one of the experiments of September 14, in which two fish were injected with 10-20 u. of insulin a few days before removing them from the crib, and then daily for a further period of four days, during which they were

Table X.—Insulin.

Date.	Condition.	Weight of fish.	Per cent. of liver-weight to body-weight.	Glycogen in liver	Blood sugar.	Hæmoglobin.
Sept. 14	10 u -20 u daily for 4 days; also previously in pen	Grms 255 0 461 5	1 8 4·6	Grms none —	Per cent. 16 8	33* 27
Sept 14	10 u -20 u daily for several days, then 10 u, then asphyxia for 3 hrs	472 439 526·5 375	4 8 3 0 3 1 4 0	— 0·486 0·107 0·195	72 123 60 81	— 30 16 30
Sept. 13	5 u insulin at 9 a.m., asphyxia 10.30-12.30	628 5 489 5	0 1 5·7	1 500 0·400	63 101	— —
Sept. 13	10 u. insulin several days previously in crib Then 10 u. a day for 3 days in lab Asphyxiated 11.15-1.15, insulin given 11.30	389 5 537	— 3 2	— trace	80 70	— 20
Aug 5	10 u on day previous asphyxia, 3½ hours	397 439	33 33	— —	121 72	— —
Aug 6	10 u 2 days previously asphyxia, 2½-3½ hours	269 595 340	— — —	— — —	56 153 96	— — —
Aug 8	10 u. 4 days previously asphyxia, 3 hours	439 539	— —	— 0·03	150 12	— —†

\* Also given insulin few hours before bleeding.

† Liver cirrhosis.

kept in rapidly changing water in the laboratory. The blood sugars of 16 and 8 mg. per cent. that were found are at the lower limits of the normal range.

To demonstrate any effect of insulin it was necessary to subject the fish to some condition which would cause hyperglycæmia and then to see whether insulin could influence it. Asphyxia was chosen as the agency causing hyperglycæmia, and it will be seen from Table X that insulin has only a slight and uncertain influence on it. Thus, if we compare the observations of Table X with those of Table II (omitting in both cases fish with liver-weight ratios of less than 2·5) it is seen, in one of the experiments of September 13, that asphyxia for two hours raised the blood sugar to 63 and 101 mg. when the insulin was injected two hours previously, and in another observation on the same day, when the insulin had been given on several days previously, to 70 and 80 mg.

per cent. These figures do not differ essentially from those obtained on normal fish asphyxiated for two hours, viz., 232, 34, 81 and 57 (Table II). When the comparisons are made for fish asphyxiated for from three to three and a half hours, we find in the insulin-injected fish (all injected frequently during several days preceding the asphyxia) 72, 123, 60, 81, 121, 72, 56, 153, 96 and 150, average 98.4 mg. per cent., as compared with 131, 168, 92, 181, 110, 136, 157, 121, average 137.0 mg. per cent. in uninjected fish. If we base our conclusion on the averages of the results there is some evidence that insulin hinders the development of asphyxial hyperglycæmia, and this is supported when we compare the maximum and minimum values in the two groups; thus, in uninjected fish there are max. 168 and 92, and in injected fish 153 and 56 mg. per cent. The hypoglycæmic action of insulin is comparatively feeble in fish.

*The Effect of Removal of the Principal Islets (Isletectomy).*

The sculpin is probably the only readily available inshore fish in which the principal islets are sufficiently isolated from the pancreas to make it possible to excise them, leaving the pancreas itself intact. By microscopic examination of sections of the pancreatic tissue in this fish both Slater Jackson and W. C. M. Scott have reported occasional small islets, but when their total mass is compared with the mass of tissue represented in the principal islets it must be almost insignificant. The operation of isletectomy was performed as follows:—

The fish were wrapped in towels soaked in sea-water, leaving the abdomen exposed. With a sharp scalpel an incision was made through the skin and superficial layer of muscle. The upper limit of this incision was about 10 mm. from the right pectoral fin, and it extended caudad for about 50–60 mm. Frequently a small blood vessel was wounded in making the incision, but bleeding was immediately stopped by ligation. The deep layer of muscle, including the peritoneum, was cautiously opened, particular care being taken not to wound the intestines, which, by being often somewhat distended, are liable to protrude. Ligatures were then passed through the abdominal wall on each side of the wound which was caused to open by gently pulling on them. By retraction with the handle of a scalpel and the little finger it was then an easy matter to expose the large islet which lies on the duodenum just caudad to the pyloric cæca. It was picked up by a fine dissecting forceps, a thin ligature tied around its base and the gland excised. Sometimes several small islets lie in a cluster in the immediate neighbourhood of the large one. These, when present, were also mass-ligated and excised. The intestines



The white columns give the results for isletectomised fish, the black columns for the operated controls. Further details with regard to glycogen and fat in the liver, weight of fish, etc., are given in Table XI. In Table XII are given the operated controls, the asphyxia controls being shown in Table VIII.

In the results for the five days following the operation the difference between the two groups is not conspicuous, although the average for the isletectomised fish on each of the days is higher than the controls. Thus, the sugar in mg. per 100 c.c. blood is as follows :—

<i>Controls</i>	<i>Isletectomised.</i>
1st day—202, 201, Av. 201.5	1st day—456, 250, Av. 353
2nd day—63, 135, 212, 343, 203, 45. Av. 172	2nd day—359, 297, 220, 229, Av. 276
3rd day—127, 250, 292, Av. 223	3rd day—290, 290, 243, 446, Av. 317
4th day—53, Av. 53	4th day—490, 298, 406, 275, Av. 367
5th day—367, 67, Av. 217	5th day—263, Av. 263

The high level of the controls up to five days is no doubt mainly dependent on asphyxia, but it is possible that the actual opening of the abdomen, the slight hæmorrhage and the irritation of the stitches might also tend to cause hyperglycæmia. It was to allow for these possible factors that the controls of Table XII were run. The value of 0.239 per cent. observed in one of the operated controls, removed on the eighth day following the replacement of the fish in the crib, is very difficult to account for. There are certain details of this observation, however, which are noteworthy. In the first place, the blood was very thin and contained only 15 per cent. of hæmoglobin. It is a curious fact, that in the vast majority of all the fish examined, in which the hæmoglobin was below 20 per cent, high sugar levels were found to occur. It is also important to note that this fish, after removal from the pen, was probably kept for more than an hour before being bled—at least the notes indicate that the first fish to be used on this day was bled at 9.30 a.m. whereas this one was not bled until 10.45 a.m. This indicates the possibility that asphyxial hyperglycæmia may have been induced and so account for the high blood sugar.

After the fourth day following the operation the control asphyxiated fish (*cf.* Table VIII) were unfortunately not so numerous as those that were isletectomised, the reason being that owing to an accident many of the former escaped from the crib. There were nine of these controls with blood sugars that were well within the normal range, but there was one fish, on the fifth day, in which 367 mg. was found. It is a curious coincidence that this fish was bled on the same day as No. 260, and it is possible that, after removal from the crib, both were inadvertently kept for too long in stagnant water. In any

Table XI.  
*Isletectomies.*

No	Date of isletectomy.	Days after isletectomy.	Blood sugar per cent.	Hæmoglobin.	Fish weight	Per cent. of liver-weight to body-weight	Glycogen in liver	Fat in liver	Remarks.
			Mgs	Per cent	Grms		Per cent	Per cent	
101	August 15	1	456	—	255	2.9	—	—	<i>P. M.</i> Small piece splenic islet remaining possibly not tied. <i>P. M.</i> Wound open, intestines escaped.
129	" 15	1	250	—	184	2.2	5.44	—	
109	" 14	2	359	30	212	2.3	5.92	—	
134	" 14	2	297	35	212	2.3	6.64	—	
102	" 16	2	220	20	142	2.4	0.70	—	
215	" 21	2	229	30	283	3.6	5.7	—	
145	September 2	2	113	30	387	4.8	4.9	—	<i>P. M.</i> Portion splenic islet left <i>P. M.</i> Small mesenteric islet left Islet in mesentery
104	August 15	3	290	35	—	—	0.15	—	
122	" 15	3	290	—10	255	3.2	0.06	—	<i>P. M.</i> Two small islets left
192	" 15	3	243	22	567	4.7	0.8	—	
193	" 15	3	446	27	299	3.6	2.50	—	
277	" 22	3	167	10	425	2.5	0.170	31.2	
247	" 19	4	298	30	—	—	0.24	—	
270	" 19	4	406	—10	439	2.8	3.12	—	
293	" 21	4	275	23	241	1.3	0.016	—	
258	" 21	4	490*	28	454	3.7	3.44	—	<i>H. B.</i> Below 10 per cent. * Minimum.
139	" 15	5	263	30	312	2.3	0.14	—	
242	" 19	6	343	28	326	2.8	1.26	20.24	
185	" 29	6	418	30	387	4.7	—	27.12	
159	September 2	6	450	18	430	4.8	2.30	—	
117	August 29	7	276	13	509	3.7	—	42.0	
162	September 4	7	266	23	521	4.0	—	—	
183	" 4	7	270	25	610	4.0	—	—	
151	August 30	8	185	35	311	4.2	0.19	43.0	
165	September 4	8	302	8	402	3.1	0.14	29.4	
157	August 29	9	166	18	653	4.4	1.34	37.4	
118	" 31	9	250	—10	298	3.6	—	—	
196	" 31	10	148	—	286	4.0	0.3	21.8	Operation difficult.
146	" 31	11	215	25	476	5.4	0.33	35.3	Fluid in abdomen, may be mixed with blood.
140	September 2	11	145	25	381	5.5	0.56	54.0	
259	August 19	16	trace	15	231	2.7	0.54	19.4	

Table XII  
*Controls (Operated).*

No.	Date of operation.	Days after operation	Blood sugar per cent	Hæmo-globin	Fish weight	Per cent of liver-weight to body-weight	Glycogen in liver.	Fat in liver	Remarks.
188	August 17	1	Mgs 202	Per cent 20	Grms. 283	2.4	Per cent. 6.24	—	
189	" 16	2	135	26	283	2.9	8.00	—	
196	" 17	2	242	26	553	5.6	13.00	—	
263	" 18	2	343	20	354	6.1	9.7	—	
245	" 18	2	203	38	198	2.5	0.77	—	
222	" 21	2	63	28	453	3.4	7.6	—	One islet removed.
187	" 17	3	250	30	425	5.2	12.24	—	
121	September 6	3	292	25	231	2.7	3.56	15.5	
130	" 6	6	trace	—	215	2.3	0.19	15.2	Fish almost lifeless when operated on.
260	August 17	8	239	15	312	2.9	2.9	—	



case the blood sugar in the highest of the controls after the fifth day is decidedly lower than the majority of the isletectomised fish. Of the latter, there are in all fourteen in which the times of survival are known, and in all of them, with one exception, hyperglycæmia of marked degree existed. This exception occurred in the case of a fish that survived the operation for sixteen days, when it was found with the abdominal wound considerably opened and the abdominal cavity filled with water, so that the low percentage of hæmoglobin (fifteen) may have been due to accidental dilution of the blood which was removed from the pericardium after puncturing the bulbus.

It will be observed, especially on examining the chart, that the hyperglycæmia became less and less marked as the interval following the isletectomy became greater. This does not appear to be related to exhaustion of the glycogen reserves.

Besides the foregoing there were three isletectomised fish which lost their tags. One of these found on September 8, which was four days since the last isletectomy, gave a blood sugar of 347 mg. per cent., and another removed on September 12, which was eight days, gave 440 mg. It is possible that both of these had been isletectomised for longer periods. Another untagged fish was removed on August 23, but since fish were being operated upon every day about this period it is impossible even to guess at the date of operation. This fish was taken because it was found in a moribund state in the pen with the abdominal wound partly open and much corrosion of the skin. After removal of the very watery blood from the bulbus, examination of the abdominal contents revealed rupture of the bile duct and evidence of peritonitis.

Taking the results as a whole, there can be no doubt that marked hyperglycæmia was set up as a result of the removal of the principal islets. That exceptional results were occasionally obtained, especially among the controls, does not seriously detract from this conclusion, since it has been shown in the first part of this paper that there yet remains, apart from asphyxia, some unknown factor which influences the blood sugar level. It is possible that this may be muscular effort. The great majority of sculpin, as far as could be observed, were seen to remain practically stationary when the crib was undisturbed and only to move when excited by the landing net or, for a few minutes, after hoisting up the crib. Occasionally a fish would be seen to be constantly swimming about, however, and it is possible that in such a fish the blood sugar was above normal. We propose to investigate the possibility further. Meanwhile, it is of interest to remark that the normal blood sugar of brook trout has been found by Noble and one of us to be much higher than in the

sculpin (0.100 mg. per cent.), these being much more active fish since they have to be constantly swimming against the stream.

*The Effect of Isletectomy on the Amounts of Glycogen and Fat in the Liver.*

Further evidence that the islets exercise a profound control over the metabolic processes is furnished by comparison of the amounts of glycogen and fat in the liver. Both substances were determined in a considerable number of cases, and for this purpose the pieces of liver after removal from the alcohol were mopped with filter paper and divided into two equal portions—one for determination of glycogen and the other for determination of fat. For this purpose the latter portion was heated on the boiling water bath, with saturated KOH solution and one half of the alcohol in which the liver had been preserved added, after the liver had become dissolved. In some cases the mistake was made of adding all of the preserving alcohol, but the error thus incurred cannot have been very great. The Leathes' modification of the Kumagawa-Suto process was used for determining the fat. The following results for fat and glycogen from fish kept for at least seven days after isletectomy are regrouped (in order of the amount of fat) so as to show the influence of isletectomy:—

<i>Isletectomy.</i>			<i>Controls.</i>		
No.	Fat.	Glycogen.	No.	Fat.	Glycogen.
250	19.4*	0.54	166	5.7	3.30
198	21.8	0.30	168	12.8*	0.10
165	29.4*	0.14	287	14.3	trace
146	35.3	0.30	130	15.2	0.19
157	37.4	1.34	136	26.8	6.36
117	42.0	trace	142	27.6	0.05
151	43.0*	0.20	176	32.7*	0.24
140	54.0*	0.56			
Av.	35.3	0.42	—	18.6	1.46

\* All of preserving alcohol added during saponification.

There is decidedly more fat and somewhat less glycogen in the diabetic as compared with the normal liver. Since it may be objected that the data are not sufficiently numerous to justify this conclusion, we add for comparison with those of the diabetic fish, results obtained on fish which were used in various of the experiments on asphyxia, etc., and which have been recorded in the first portion of this paper. These are arranged in the order of the amounts of fat found present.

Date.	Fat.	Glycogen.	Liver-body-weight ratio
September 5	15.8*	11.2	4.4
August 26	17.4*	5.6	2.9
September 10	18.0*	12.0	3.8
August 25	22.2	7.6	4.7
August 29	22.0*	3.8	2.4
September 10	24.4	4.7	4.3
August 29	52.5	0.1	5.0
September 5	29.2*	1.5	2.6
August 29	29.7*	5.0	3.3
August 26	30.0*	5.3	2.1
August 29	31.7	2.8	4.3
September 14	36.5*	1.3	4.0
August 29	38.0	6.4	5.0
Average	26.23	5.17	

It will be seen that five out of nine of the fat values for the isletectomised fish exceed the maximum for the control fish, and six of them exceed the average for those of the last table. When it is remembered that the isletectomised fish were without food for at least six days, the contrast becomes very marked. The differences in the amounts of glycogen in the two tables are also marked, but in this case it is difficult to say to what extent isletectomy is really responsible, since several of the fish used as "controls" gave very low values. Incidentally it will be observed in the results from normal fish that there is no evident relationship between the amounts of fat and glycogen, or between these and the weight ratios. In a general way, when excess of glycogen is present there is much less fat than usual, but high percentages of both may exist side by side. When subnormal amounts of both are present fasting is the probable cause.

These results afford further evidence that the source of insulin in the animal body is the islet tissue. When this new evidence is considered along with that furnished by the fact that extracts of the principal islets contain insulin in quantities far in excess of those extractable from other tissues, including the practically islet-free pancreas of Teleostei (*Myoxocephalus*, *Lophius*) the evidence for the hypothesis that the islets are the source of insulin in the animal body would seem to be complete. That it should be possible to prepare from organs and tissues, other than the pancreas and the principal islets, extracts having insulin-like effects on normal rabbits is probably to be interpreted as indicating the storage in them of insulin transported by the blood. In any case, extra-pancreatic insulin cannot be of significance in the regulation of the metabolism of the carbohydrates, since this completely breaks down when the

pancreas is removed in mammals, but not when any other organ or tissue is removed. If it be the case that administration of insulin from other sources than the pancreas or principal islets can remove the diabetic symptoms, we must conclude that the traces of insulin said to be present in the tissues of the depancreatized animal are not available in its metabolism, possibly because they are combined with some substance so as to produce an inert compound, such as has been demonstrated by Epstein and Rosenthal (14) to be formed between insulin and trypsin or pepsin (15). It was not possible in the time available to determine whether injection of insulin into isletectomized fish would restore the blood sugar to the normal level, but since it can diminish asphyxial hyperglycemia it will probably be found to have this effect.

#### *Summary and Conclusions.*

1. The sugar in the blood of salt-water fishes immediately after catching varies considerably, both among individuals of the same species and among those of different species. Thus in *Myoxocephalus* (sculpin) it may vary from a trace to 35 mg. per cent.

2. The exposure of the fish to air, as in catching, causes marked hyperglycemia, which sets in in about 30 to 45 minutes and may cause the blood sugar to rise to 160 mg. per cent. within one hour.

3. This hyperglycemia can readily be induced by placing the fish in a limited volume of stagnant water, and in this its rate of development is accelerated by raising the temperature.

4. In two or three hours, under ordinary conditions of temperature, the sugar may rise to about 200 mg per cent., but the extent to which it does so varies considerably in different individuals.

5. Replacement of the fish in frequently changing water, either in the sea or in a properly constructed aquarium, is not followed by return of the blood sugar to the normal level until after two to four days, or occasionally longer.

6. The amount of glycogen in the liver also varies very greatly in different individuals, but it is not possible, in most cases, to correlate this with the normal blood sugar or with the degree of the hyperglycemia caused by asphyxia.

7. By hydrolysis of fresh blood with 0.1 N acid a marked increase in reducing power occurs, and a smaller increase may be detected when the protein-free filtrate is similarly hydrolysed. It is considered possible that a part of the asphyxial rise in blood sugar may depend on hydrolysis of non-reducing (masked) carbohydrates in the blood.

8. The average amount of fat in the liver of *Myoxocephalus* is 26 per cent.,

and it varies much less than that of glycogen. There is not usually a reciprocal relationship between fat and glycogen, so that the ratio of liver-weight to body-weight is no indicator of the amount of either, or both, of these reserve food-stuffs in the liver.

9. Glycolysis does not occur within 10 hours in the oxalated blood of *Myoxocephalus*, kept at room temperature, and taken from either normal or asphyxiated fish

10. Intramuscular injection of epinephrin (adrenalin) causes marked hyperglycæmia, reaching its maximum in about two hours.

11. Intramuscular injection of insulin has only a slight effect on the blood sugar. This cannot be demonstrated on normal fish because the blood sugar is already at a low level and is often nearly absent. It can, however, be detected by subjecting fish previously injected with insulin to asphyxia, when the blood sugar rises less than would be expected without insulin. These effects of insulin have been obtained both after injecting the insulin just prior to inducing asphyxia and after injecting it daily for several days preceding the asphyxia.

12. Removal of the principal islets in *Myoxocephalus* is followed by marked hyperglycæmia. In isletectomised fish examined up to the fifth day after the operation, the blood sugar was found to be considerably above that of control fish that had been exposed to air for a period of time corresponding to that of the operation, or had been operated upon without actually removing the islets. After the fifth day the differences between the two groups of fish was much more striking, the controls usually showing blood sugars within the normal range, whereas in the isletectomised fish they were increased from three to twelve times the normal.

13. From the fifth to the eleventh days following isletectomy the hyperglycæmia became steadily less marked, but it was not possible to correlate this with the amount of glycogen in the liver.

14. There was more fat and less glycogen in the livers of isletectomised, as compared with normal fish.

We are indebted to Miss Marion Armour and Miss N. R. Hearn for making most of the analyses for glycogen and all of those for fat, and to the Biological Board of Canada and its director (Dr. A. G. Huntsman) for placing the excellent facilities of their Atlantic Station at our disposal. The expenses of the investigations have been partly defrayed by grants from the Carnegie Corporation.

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*The Meiotic Phase in Triton (Molge vulgaris).*

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(Communicated by Prof J. B. Farmer, F.R.S —Received June 19, 1924.)

[PLATES 1-9.]

While it will be necessary to deal in greater detail at a later stage with some of the phenomena herein described, and with their bearing upon the work of others, I shall begin with a general description of my observations. I propose to adopt some of the terms used by Miss Digby in her paper on the "Meiotic Mitoses in Osmunda," in order to avoid confusion, and also because these observations on the Meiotic phase in Triton agree, at any rate as to some of the most important points, with hers on Osmunda.

"The term *thread* will be used to specify the longitudinal *half* of an entire univalent spireme or chromosome."

"The term *filament* will be used to specify the *entire* univalent spireme, the product of the close lateral association of two threads (*i.e.*, of two longitudinal halves of univalent spireme." I would add, or chromosome.

Beginning at the telophase of the division which precedes the last somatic mitosis before the first Meiotic (heterotype), a very definite longitudinal fission appears in the daughter chromosomes, particularly at their ends, and before the nuclear membrane of the daughter cell is formed (Plate 1, fig. 1). As the daughter nucleus develops this fission proceeds, and the threads separate more and more, until the whole nucleus is occupied by single fine semi-valent threads, which appear to become thinner and less distinct as the process advances (figs. 2-8). A stage is eventually reached when this network of fine threads is only just distinguishable (fig. 9).

I regard this stage as the end of the telophase and the beginning of the prophase of the next division.

After this the threads become clearer and thicker (Plate 1, fig. 10) and approximate in pairs, at first running parallel for short distances only (figs. 11 and 12). This parallelism gradually becomes more and more marked, and the threads approximate until the whole nucleus is occupied by a coiled and apparently continuous filament (spireme) formed by the approximation of the two semivalent threads (figs. 13 and 14). This filament breaks up into 24 chromosomes, which, after the nuclear membrane has disappeared and they have become attached to the spindle fibres, split longitudinally. The longitudinal halves travel to each pole of the spindle and form the nuclei of the two daughter cells (figs. 15-20). These daughter cells now enter upon the Meiotic phase.

The succeeding stages, up to that which I have defined as the end of the telophase and the beginning of the prophase of the next division, closely resemble those preceding the somatic division just described, except that there is a very striking and enormous growth of the whole cell, and more still, in proportion, of the nucleus (Plates 2, 3, figs. 21-25). The chromosome filaments split into semivalent threads, which become thinner and more indistinct until the nucleus appears to be in practically the same condition, though approximately half as large again in diameter, as those of the previous generation at this stage.

The threads then become more distinct, and approximate until considerable lengths are apparently fused together into filaments (figs. 26-28). At the later period of this stage some of these univalent filaments appear to be arranged parallel to each other in pairs, the parallel portions pointing towards one pole of the nucleus (Plates 3, 4, figs. 29-32). These pairs of more or less parallel filaments are again arranged, at considerable distances, parallel to each other. But this parallelism appears only towards one pole; at the other parts of the

nuclear periphery the filaments diverge. Moreover, while the threads have apparently almost fused into univalent filaments towards the pole, at the other ends they diverge, forming loops between the ends of the filaments and producing the early stage of what Janssen aptly called the "bouquet." The threads and filaments throughout this period become thicker and more and more distinct. These univalent threads approach each other and a definite conjugation takes place (figs. 33 and 34). The same apparent polarity continues. The semivalent threads looping together the ends of the univalent filaments in their turn approximate, while at the same time the filaments at their other ends are joining together in pairs to form the beginnings of bivalent loops. The process continues until the nucleus is filled with bivalent loops, the ends of which are arranged about one pole of the nuclear membrane while the curves travel through the nucleus, giving the complete "bouquet" appearance (figs. 35-37)

While these bivalent loops are thicker by far and apparently contain more chromatin than was the case at an earlier stage of the four semivalent threads of which they are composed, their structure is loose. It can readily be seen at many points that they are formed of two filaments and sometimes that the filaments are formed of two threads, but generally there are so many strands containing particles of chromatin that the whole appearance is somewhat ill-defined. The formation of the filaments is best seen in transverse section.

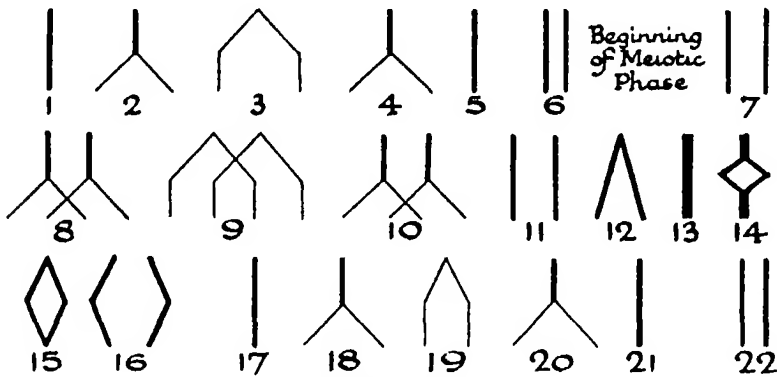
From this point the loops become much denser in structure and more sharply defined (figs. 38-40). But at the same time the conjunction between the two filaments forming each loop appears to relax, beginning generally near the centres of the loops at their greatest convexity. (*a*, figs. 38 and 39.) This continues until the pairs of conjoined filaments are completely separated except at their ends, which remain joined together (Plate 5, figs. 40-44). During part of this period it may be observed that the filaments tend to separate at some points into the two threads of which they are composed (*a*, figs. 41-43).

There are thus in the nucleus at this stage a number of pairs of univalent filaments joined at both ends to each other. There are 12 of these pairs, half the somatic number of chromosomes. These gradually assume definite shapes and form the bivalent chromosomes or Gemini of the 1st Meiotic division (figs. 45-47). At the actual division, these pairs of units of univalent filament are apparently separated at the points where they were joined, ends to ends, one unit going to one pole and thus to one daughter cell, the other to the other daughter cell. I suggest that these units are complete somatic chromosomes.



A striking feature of the anaphase and telophase of this division is that a fission of the chromosomes (filament) into the two component semivalent threads, appears to a very marked degree (figs. 49 and 50), and this fission continues until the filaments are completely separated into two semivalent threads in the later stages of the telophase of the 1st, and the beginning of the prophase of the 2nd Meiotic (homotype) division.

The processes in the 2nd Meiotic division are similar to those already described in the somatic division, except that only half the number of chromosomes is developed (Plates 6, 7, figs. 51-63). A feature of the telophase of the 2nd Meiotic division is that the daughter chromosomes and the filaments derived therefrom divide into semivalent threads, although there is no mitosis in prospect until the first segmentation after fertilisation (Plates 7, 8, figs. 64-67) Text-fig A gives a diagrammatic representation of these phases.



TEXT-FIGURE A.

1-6, Somatic division 7-16, 1st Meiotic division. 17-22, 2nd Meiotic division 6-22, represent the longitudinal splitting of the chromosomes in the Metaphase

According to the observations here described there is no fission of the individual chromosomes in the 1st Meiotic division. The cell as a whole divides into two daughter cells, and the individual chromosomes are distributed, half of them going to each of these. In every other division, both before and after the 1st Meiotic, each individual chromosome splits, a longitudinal half of each going to each daughter cell. The separation of the univalent filament in the telophase of the last somatic division is, however, never consummated until the 2nd Meiotic division, when the chromosomes split into longitudinal halves. The 1st Meiotic division therefore is a unique phenomenon interpolated between two mitoses. It provides for the equal distribution of the whole chromosomes.

In the spermatogonia an appearance is constant which suggests amitotic division very strongly (fig. 70). A recognition of the various stages of telophase and prophase of the somatic mitosis, and a comparison between them and the many-lobed nuclei of the spermatogonia has led me to believe that amitosis does not take place. The nuclei of these cells always present the appearance of one of the stages in somatic mitosis (figs. 70-74). Frequently several nuclei divide within a common cytoplasm, but I have never observed a pluripolar mitosis.

Certain cytoplasmic structures seem to be constant, in the latter part of the Meiotic phase at any rate, though I have not sought them carefully in the preceding generations.

In the cells preparing for the 2nd Meiotic division a number of small vesicles can sometimes be observed in the archoplasm (*a*, figs 57 and 59). These reappear in the spermatids (fig 65). They can sometimes be traced through the 2nd Meiotic division itself (fig 64). In the spermatids these vesicles coalesce and form a large vesicle destined to form the cap (acrosome) of the sperm (figs. 66-69).

#### DISCUSSION OF DETAILS

##### *The Somatic Division.*

It seems to be widely accepted that the daughter chromosomes in the telophase of the somatic division split, and that the halves form threads which subsequently rejoin to form the Spireme (Grégoire and Wygaerts, 1904; Grégoire, 1906, Digby, 1910, Frazer and Snell, 1911). Grégoire (1913), however, says that this is not a true division of the daughter chromosomes; why he comes to this conclusion I do not understand. Any long discussion upon this series of phenomena seems unnecessary. The circumstance which strikes one is that some of the recent authors do not go far enough back in their references. Flemming (1891) described a longitudinal division in the daughter chromosomes in the telophase in Salamander, and called it "precocious longitudinal division." This observation was confirmed by Brauer (1892) and by Reinke (1895). These authors, however, seemed to attach but little importance to the phenomenon. The first who treated it as being of fundamental importance in animal cells, so far as I have been able to ascertain, was Dehorne (1911). He, after describing the splitting of the daughter chromosomes into two threads, describes these threads as splitting again, and as I understand him, makes the number of chromosomes double that

which appears in the metaphase of the somatic division. Each somatic chromosome is, in fact, according to him bivalent. I have been unable to find any trace of this second splitting. Grégoire (1912) denies that it occurs.

The obvious interpretation, which has been generally adopted, is that the fission observed in the chromosomes in the telophase is the precursor of the longitudinal division of the chromosomes in the subsequent metaphase and anaphase.

In many animals and plants the nuclei of the cells are described as passing through a period of "rest." This has been stated to occur in the spermatogonia and spermatocytes of Triton by several authors (*e.g.*, Janssens, 1901; Moore and Embleton, 1906; Champy, 1913). I take it this means only that the nuclei remain in one particular stage longer than in any other. Judging by the figures published in the past, the stage generally regarded as the "resting" stage is that shown in my fig. 4 (Plate 1). This is also very similar to the figures generally given of the "resting" nuclei of the tissue cells in Triton. According to my present observations the stage is a lengthy one, as frequently there are more nuclei in this condition than in any other in a given section. I would point out, however, that it is sometimes difficult to discriminate between this stage and that illustrated in fig. 11 (Plate 1), though the size of the nucleus is generally a guide.

It is somewhat remarkable that the prolongation of a particular stage should not occur at what is apparently the end of the telophase and the beginning of the prophase.

Towards the end of the telophase and at the beginning of the prophase (figs. 7-10) the nuclear substance appears to be more chromatic, but except in the earlier stages of the telophase there does not appear to be any polarity in the arrangement of the chromatin. The number of somatic chromosomes is 24.

### *The 1st Meiotic Division.*

In the telophase of the last somatic division the splitting of the chromosomes into semivalent threads takes place just as in the previous division. In the prophase of the 1st Meiotic division these threads associate again as univalent filaments as in the preceding somatic prophase. It is therefore remarkable, as Miss Digby points out (1919), that Grégoire and his followers, while supporting these observations with regard to the somatic division, regard the pairing of the semivalent threads in the 1st Meiotic prophase as the coming together of univalent filaments and not of threads (Grégoire, 1907).

In Triton, these stages are particularly clear, and there can hardly be a doubt that in both the somatic and 1st Meiotic divisions the splitting of the chromosomes, and filaments derived therefrom, into semivalent threads and the re-joining of the threads to form univalent filaments, are identical processes.

Several authors (*e.g.*, Janssen, 1901 ; Champy, 1913) have apparently taken what has generally been described as the "resting" stage in Triton for the beginning of the prophase, and I think that this has misled them in their interpretation of the subsequent stages. They both regard the threads as univalent filaments and describe them as pairing, shortening and thickening, as do many other authors in other animals. (Lerat, 1905 ; Van Molle, 1907 ; Agar, 1911). In many forms this interpretation has been attributed, probably rightly, as due to the fact that the subsequent pairing of univalent filaments during synapsis has been missed (Digby, 1919). In Triton there is no synapsis. This fact was recorded by Janssens (1901) and I am able to confirm his observation most emphatically. My own experience leads me to agree with him that the apparent synapsis, sometimes described as occurring in Triton, is entirely due to imperfect fixation or to subsequent faulty treatment of the material. It appears to me to be a mistake to apply the term synapsis to the stage figured as such by Moore and Embleton (1906), which seems to correspond to my fig. 22. In their figures there is no sign of contraction or bunching in the filaments or loops. I have failed to find the definite number of rod-like bodies described by them in the "resting" nuclei.

I believe that the pairing of the semivalent threads and the subsequent pairing of the univalent filaments have been missed in Triton because these two stages overlap (figs. 30-33), and also because, judging by the infrequent occurrence of nuclei showing the critical phase (fig. 33), the process is very rapid.

It has already been briefly stated that, as the filaments are formed at one pole by the union of the threads, they are arranged parallel to each other and gradually coalesce in pairs. As the process begins at one pole and proceeds gradually, one finds the beginnings of bivalent loops at the pole, grading off through univalent filaments to semivalent threads. Precisely the same result appears to be produced as described in *Osmunda* and *Doodia* (Digby, 1919 ; Sarbadhikari, 1924), during the first and second contractions, but the process in Triton appears to be less complicated and is more easy to observe owing to the absence of any synapsis. Both in their observations and my own, the semivalent threads coalesce in pairs to form univalent filaments, and then the filaments coalesce in pairs to form bivalent loops or spireme.

Owing to the large size of the cells of Triton, more particularly of those

passing through the 1st Meiotic division, the nuclei of which are generally nearly  $20\ \mu$  in diameter during the bouquet stage, it is difficult to get a whole nucleus in a section thin enough for accurate observation; however, the number of loops appears to be 12—that is, half the somatic number of chromosomes.

The sequence of events after the formation of the complete "bouquet" as described earlier in this communication, does not, I think, raise any seriously controversial point. The main differences from some other observers are the partial division of the filaments into threads at the stages illustrated at *a* in figs. 41 and 43, and the continued adherence to each other of the somatic chromosomes in pairs by their ends after separating from each other throughout their whole length.

The partial separation of the threads in the filaments at this stage is not surprising, if the validity of the interpretation of the previous and subsequent stages is accepted. The mode in which the somatic chromosomes separate appears obviously consequent upon the preceding stages.

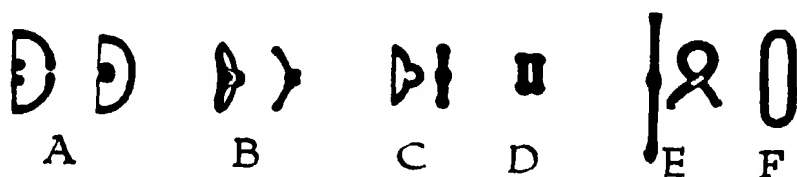
Agar (1912) describes the appearance of fine threads during the 1st Meiotic prophase in *Lepidosiren*. He states that these appear shortly after the resting stage, which I am inclined to think from his figures corresponds to the stage in *Triton* illustrated by my fig. 29, as his fine-thread stage (I think) corresponds to my fig. 30. He, however, describes no further pairing of the loops, but states that the threads go into synapsis and separate again. He describes the formation of rings through the adherence of the chromosomes to each other by their ends, in a similar manner to that described here as occurring in *Triton*. I have, however, observed nothing similar to the complete separation of the somatic chromosomes from each other and their subsequent pairing again which he describes. He mentions the split in the somatic prophase, but neglects the telophase, both in the earlier somatic divisions and in that immediately preceding the prophase of the 1st Meiotic division. These are of fundamental importance if Miss Digby's observations and those given here are correct.

It will be noted, on referring to the illustrations, that from a very early stage in the prophase of the 1st Meiotic division there is a definite polarity in the arrangement of the chromatin threads, which polarity increases and culminates in the "bouquet" stage. The chromatic appearance of the nuclear substance generally during the late telophase and early prophase is similar to that observed in the corresponding stages of the somatic division (figs. 22–27).

In the complete "bouquet" stage it is very difficult, and in the majority

of cases impossible, to make out the composition of the loops. They are very ragged in appearance and send out innumerable processes and strands containing chromatin granules. Only when cells are chosen which show transverse sections of the loops can it generally be seen definitely that these are built up of two, more rarely of four, components (fig. 37).

The 1st Meiotic gemini or double chromosomes in Triton assume certain definite shapes, as observed by Moore and Arnold (1906). I can confirm their observation that these definite forms are apparent before the nuclear membrane disappears. Moreover, I am able to add that these forms may frequently be seen in course of gradual formation (a, fig 45). The text-fig. B shows the



TEXT-FIGURE B

forms I have observed in the Meiotic Gemini of Triton (*Molge vulgaris*). They correspond almost exactly with those given by Moore and Arnold, and the slight differences may be due to the fact that possibly I have not dealt with the same species, as they do not give the species of Triton used by them. There are apparently always two of each shape in Triton. As I have pointed out, however, on another occasion (Walker, 1911), these shapes, though so striking and definite as to make confusion between any two of them very improbable, vary within considerable limits, both as to details in shape and as to size. I have given profile as well as full views of the shapes where desirable, but it must be remembered that the chromosomes may present themselves from any point of view, and if seen from a pole of the spindle it is practically impossible to discriminate between some of them.

#### Absence of Amitosis

Several observers, including myself (Walker and Embleton, 1906), have stated that amitosis occurs in the early spermatogonia of Triton. A further study in the light of more recent observations has led me to believe that this is a mistake. Some of the cells present an appearance which suggests direct division very strongly (fig. 70). A comparison with the figures illustrating the telophase and prophase of the somatic division shows, however, that these

nuclei are, as far as I have been able to ascertain, always in an active stage of one of these processes. It is probable that the process is hurried over in the metaphase, for this stage is not often to be seen. I note that Child (1907-11) after a controversy with Richards (1909-11), though not withdrawing from his statement that amitosis does occur in the direct line of cell generations producing the gametes in the forms he studied, modifies his position and allows mitosis to be more frequent than in his original observations. As he gives mere outlines of cells and their nuclei in his figures, it is difficult to criticise his observations.

#### *Archoplasmic Vesicles.*

Vacuoles and other bodies have been described as giving rise to the cap or acrosome of the spermatozoon. Meves (1899) calls the body "Bläschen," Benda (1896) "Vacuole." Baumgartner (1902) observed it, but only in a late stage in the spermatid. Small vesicles have been described as occurring in the archoplasm of the cells during the 1st Meiotic division, disappearing and reappearing in the archoplasm of the 2nd Meiotic division, and again in the spermatid in Mammalia (Moore and Walker, 1906). In the spermatid these vesicles coalesce into a larger vesicle which grows, its membrane embracing the nucleus, while the larger end forms the cap. Montgomery (1910) describes the body from which the cap arises as dividing into two, one part forming the cap, the other disappearing.

More recently Doncaster and Cannon (1920) describe the acrosome in the louse as arising from a spherical deeply-staining body about the size of the nucleolus. According to them, however, it is of extraordinary variability in staining capacity and often is not demonstrable. They do not describe it as a vesicle at any time. The methods they used suggest that the fixation was poor in some cases. Bowen (1922) figures these vacuoles in certain insects, but does not refer to them. According to his figures they apparently disappear.

Gatenby and Wooger (1921) derive the acrosome in the guinea-pig from small bodies which they call "proacrosomic granules," and which they claim to trace through the Meiotic divisions, as also do Papanicolaou and Stockard (1918). It is, however, only at a comparatively late stage that Gatenby and Wooger describe these bodies as vesicles. They say that the "proacrosomic granules" have formed round them in the spermatid a liquid-filled space, "so that it comes to lie in an archoplasmic vacuole." After this they describe the production of the acrosome in much the same way as has been recognised by many observers during the past twenty years or so. Their figures and

description suggest that the preservation of the material may have been in fault.

In well-treated material the bodies that eventually produce the acrosome in the guinea-pig appear in the archoplasm as definite vesicles with darkly staining granules in their centres (Moore and Walker, 1906). If the "proacrosomic granules" represent the archoplasmic vesicles under a new name, which is possible as they are described as occupying a similar position, it is probable that, owing to the drastic treatment to which the material had been subjected, the vesicles had collapsed and presented the form of granules. Gatenby and Wooger connect these bodies with the Golgi apparatus, and state that they are not demonstrable by the generally accepted cytological methods.

The usual fixatives and subsequent treatment of material for cytological investigation have been adopted through long experience and comparison of the effect upon structures which can be observed in the living cell, and by the constant and similar or changed appearance of like bodies under different treatment. It is common knowledge that certain reagents produce more or less distortion or even destruction of cell structures. Maceration, heat, drying and other methods of treatment are as fatal to good preservation as unsuitable reagents. Certain methods of staining are likely to produce artifacts. I have tried some of the less drastic of the methods used by Gatenby and Wooger, and the effect upon the well-known structures, which can be observed in the living cell, is such as to make me unwilling to accept their statements as to the existence of bodies which can be demonstrated only by an extreme treatment of the material. From some of the figures I see but little reason, except sometimes on account of their positions, for connecting any of the irregular and widely dispersed granules shown with the archoplasmic vesicles. It is only in the later stages, when the vesicles have coalesced and the single vesicle has grown to a considerable size and reached a stage of differentiation sufficient to resist severe treatment that the precursor of the acrosome becomes recognisable in these figures. The vesicles with a granule in each were demonstrated nearly 20 years ago in the guinea-pig in the archoplasms of the cells of the 1st and 2nd Meiotic generation and in the spermatids, strong Flemming, Heidenhain and acid fuchsin being used (Moore and Walker, 1906). I have here traced the vesicles through the 2nd Meiotic division in Triton with the same methods.

A significant remark in relation to the question of fixation and other methods is:—"The periods of division of the spermatocyte (in guinea-pig) are difficult properly to study. In very little of our material were mitoses to be found"



(Gatenby and Wooger, 1921). I have examined hundreds of sections of the testes of guinea pigs at frequent intervals during the past 20 years, and have prepared new specimens fairly regularly. Never, in a properly preserved specimen of a normal adult testis, have I had any difficulty in finding division figures among the spermatocytes of both the 1st and 2nd Meiotic generations.

I have not followed the development of the acrosome and spermatozoon further, as it is not the main object of the present investigations, nor have I tried to trace the archoplasmic vesicles in the first Meiotic generation of cells.

### *Methods and Material.*

The testes of *Molge vulgaris* were taken generally twice, rarely only once, a week, from April 1 until August 9. Portions not more than 1 mm. thick were placed in the fixative within one minute of the animal being decapitated. The fixative that proved most satisfactory was strong Flemming: Zenker's ordinary fluid and one with the addition of copper sulphate; acetic, corrosive and alcohol, were also used. The tissue was never subjected to a temperature higher than 45° C. All specimens were imbedded in paraffin, fixation and dehydration occupying about three hours, imbedding about one and a half to two hours. The stains used were Heidenhain's iron-alum and hæmatoxylin, sometimes counter-stained with acid fuchsin and orange G, saffranin in 85 per cent. alcohol; basic-fuchsin, methylene blue and orange G. Some other stains were used, but these proved most useful. The staining with Heidenhain was most satisfactory when the whole process was carried out in less than one hour.

The Meiotic divisions were observed first in the specimens fixed on May 18. They were numerous in the specimens obtained on June 8, and continued numerous until July 13. Champy (1913) gives the time of coupling of this species as April, of spermatogenesis as end of July to September. My specimens evidently developed earlier than this. Apparently the spermatozoa are stored through the winter.

I wish to express my thanks to Prof. J. B. Farmer and Mr. Clifford Dobell for helpful criticism and assistance with regard to references. I am indebted to Miss Digby's paper on *Osmunda* (1919) as having suggested this investigation.

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## DESCRIPTION OF PLATES 1-9 (Figs 1-75).

N.B.—The magnification is not the same in all the figures. A scale is given with each.

Figs. 1-9 are rather more magnified than those immediately following.

FIG. 1.—Telophase of somatic division showing splitting of daughter chromosomes.

FIGS. 2 and 3.—Later stages showing the splitting of the univalent filaments into semivalent threads.

FIGS. 4-7.—Progressive separation of filaments into threads. Chromatic appearance of nuclear substance becoming evident.

FIG. 8.—Completion of the separation of filament into threads.

FIG. 9.—End of telophase and beginning of prophase. Threads indistinct.

FIG. 10.—Threads again becoming distinct. Individual portions parallel at a few points.

FIGS. 11 and 12.—Parallelism of threads increasing. They are becoming thicker and apparently shorter.

FIG. 13.—Approximation of threads to form univalent spireme nearly complete.

FIG. 14.—Spireme.

FIG. 15.—Spireme separated into chromosomes.

FIG. 16.—Disappearance of nuclear membrane.

FIGS. 17-20.—End of prophase, anaphase, early telophase of last somatic division. The daughter chromosomes show longitudinal fission.

FIGS. 21-24.—The univalent filaments separating into semivalent threads. Considerable growth of cell.

FIG. 25.—End of telophase and beginning of prophase of 1st Meiotic division. Growth of cell and particularly of nucleolus continues.

FIGS. 26-29.—Threads become thicker and shorter and approximate as in the preceding somatic prophase. Polarity in arrangement of threads is evident.

FIG. 30.—Early "bouquet" stage. The parallel pairs of threads are approximating to form univalent loops. Polarity more marked.

FIG. 31.—Filaments, where they have formed, are arranged parallel to each other. Threads and filaments becoming thicker.

FIG. 32.—Cell showing same stage but filaments in transverse section.

FIG. 33.—Univalent filaments joining to form bivalent loops.

FIGS. 34-36.—Further progress of conjunction of filaments. Complete "bouquet" stage. Shortening and thickening of loops is marked.

FIG. 37.—Transverse section of loops in complete "bouquet" stage. The composition of the loops from two filaments or from four threads can sometimes be seen.

FIG. 38.—The bivalent loops are beginning to separate into their component univalent threads as at *a*. Chromatin more dense in arrangement.

FIGS. 39 and 40.—Further progress of separation of filaments.

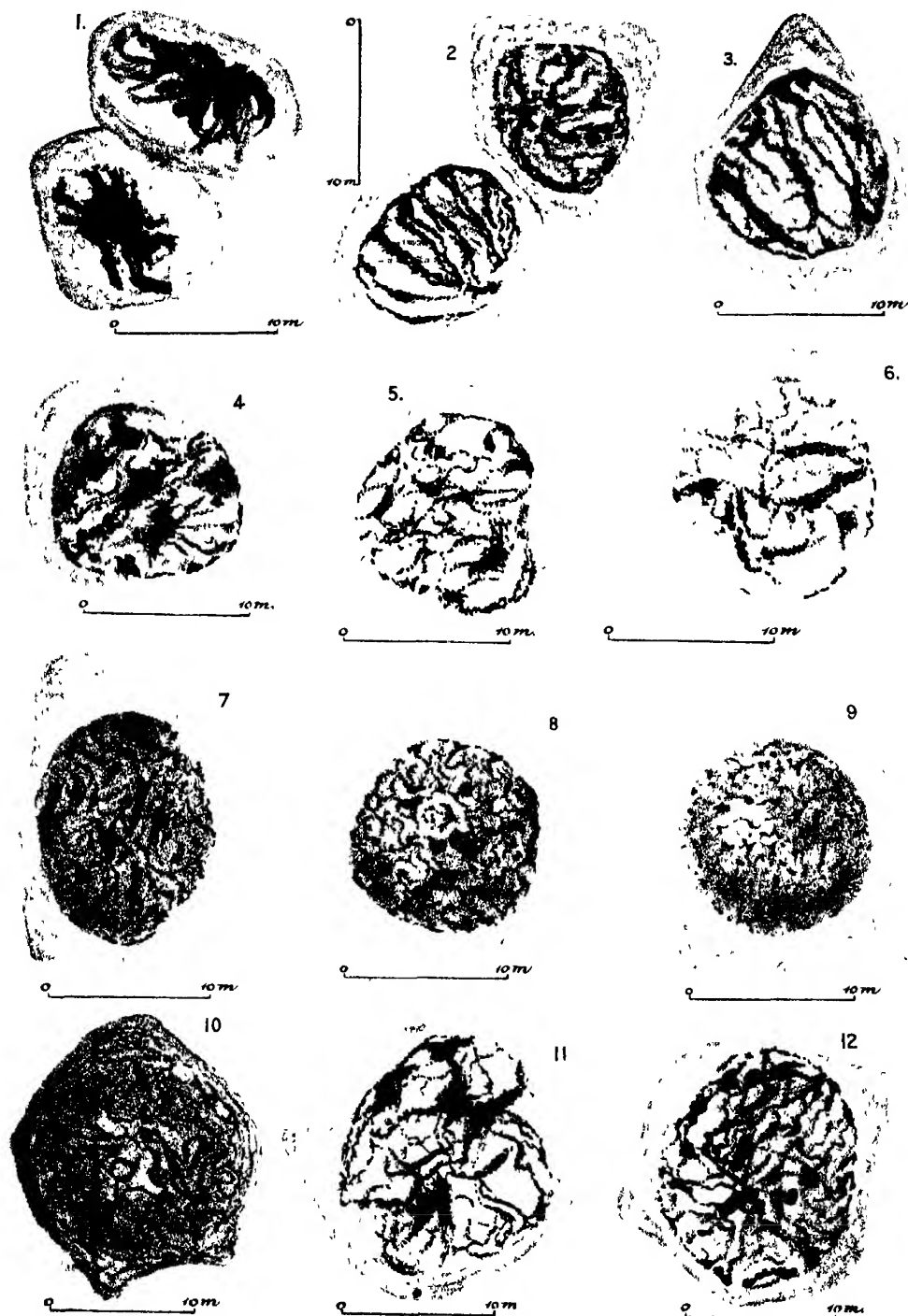
FIG. 41.—Separation of filaments further advanced. At some points (*a*) they have divided into semivalent threads.

FIGS. 42 and 43.—Further separation of filaments to form irregular rings.

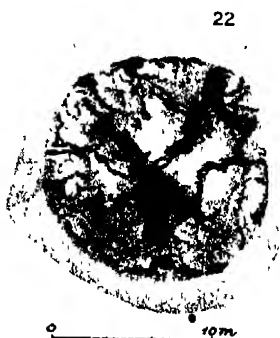
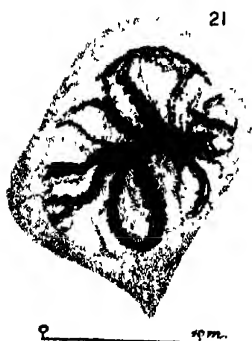
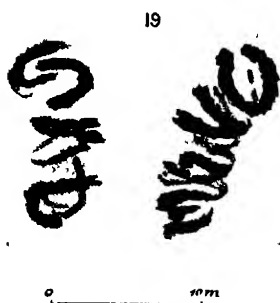
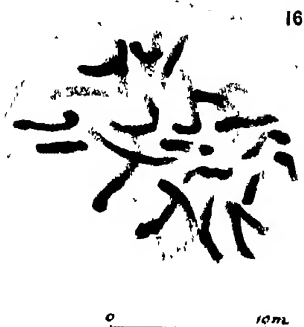
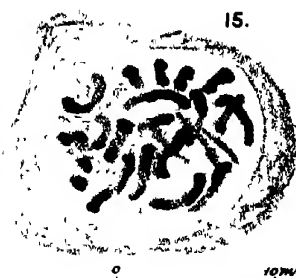
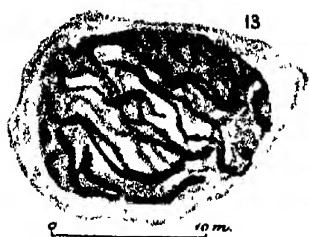
FIG. 44.—The filaments have separated completely except at their ends. All signs of fission into threads has disappeared.

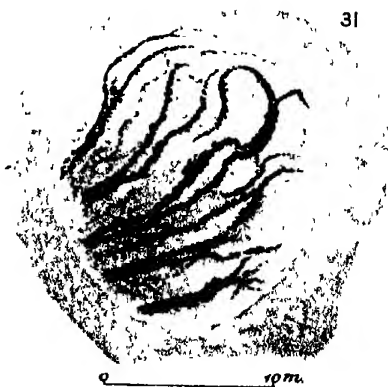
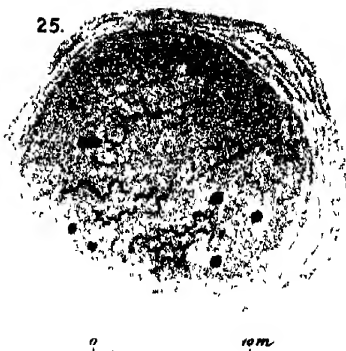
FIG. 45.—The rings have become shorter and denser. They are beginning to assume the shapes of the first Meiotic gemini *a*. (This is the shape represented at B in text-fig. B.)





*C.E Walker, del.*



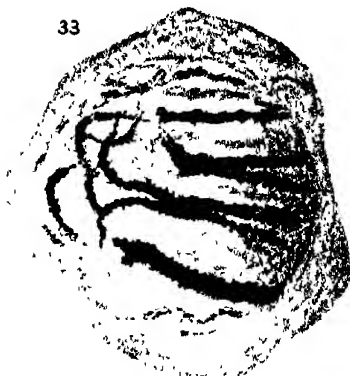


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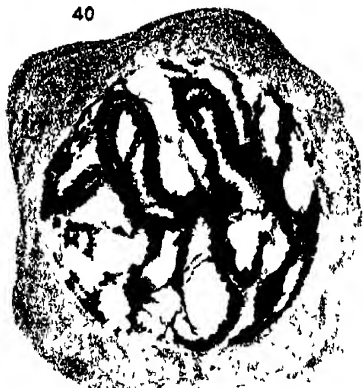
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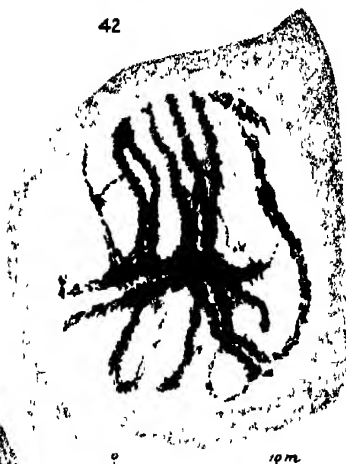
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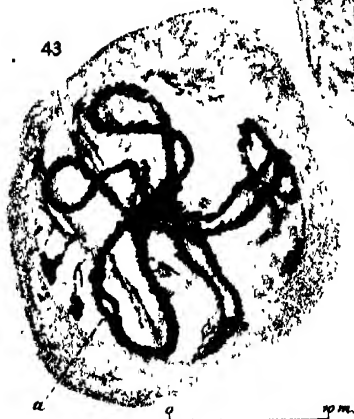
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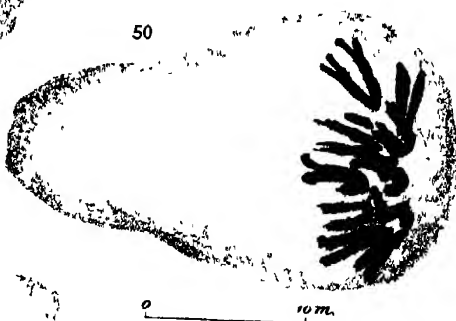
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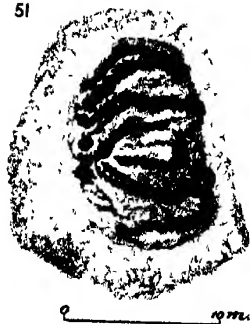
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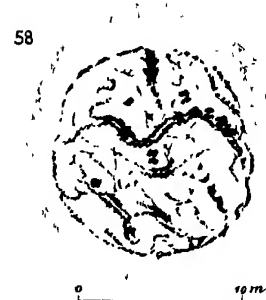
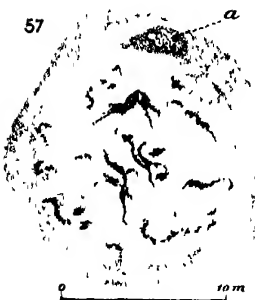
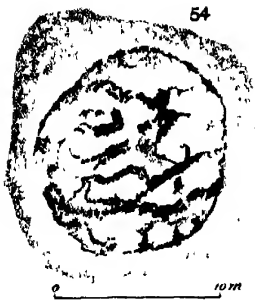
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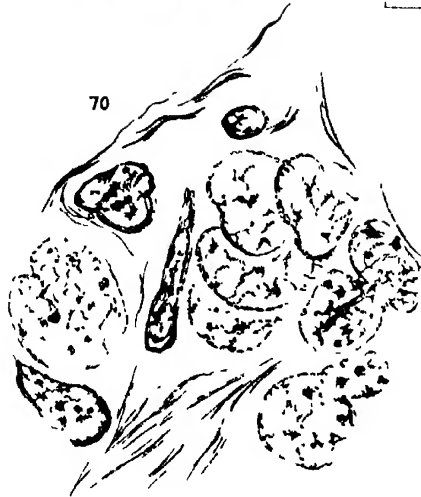


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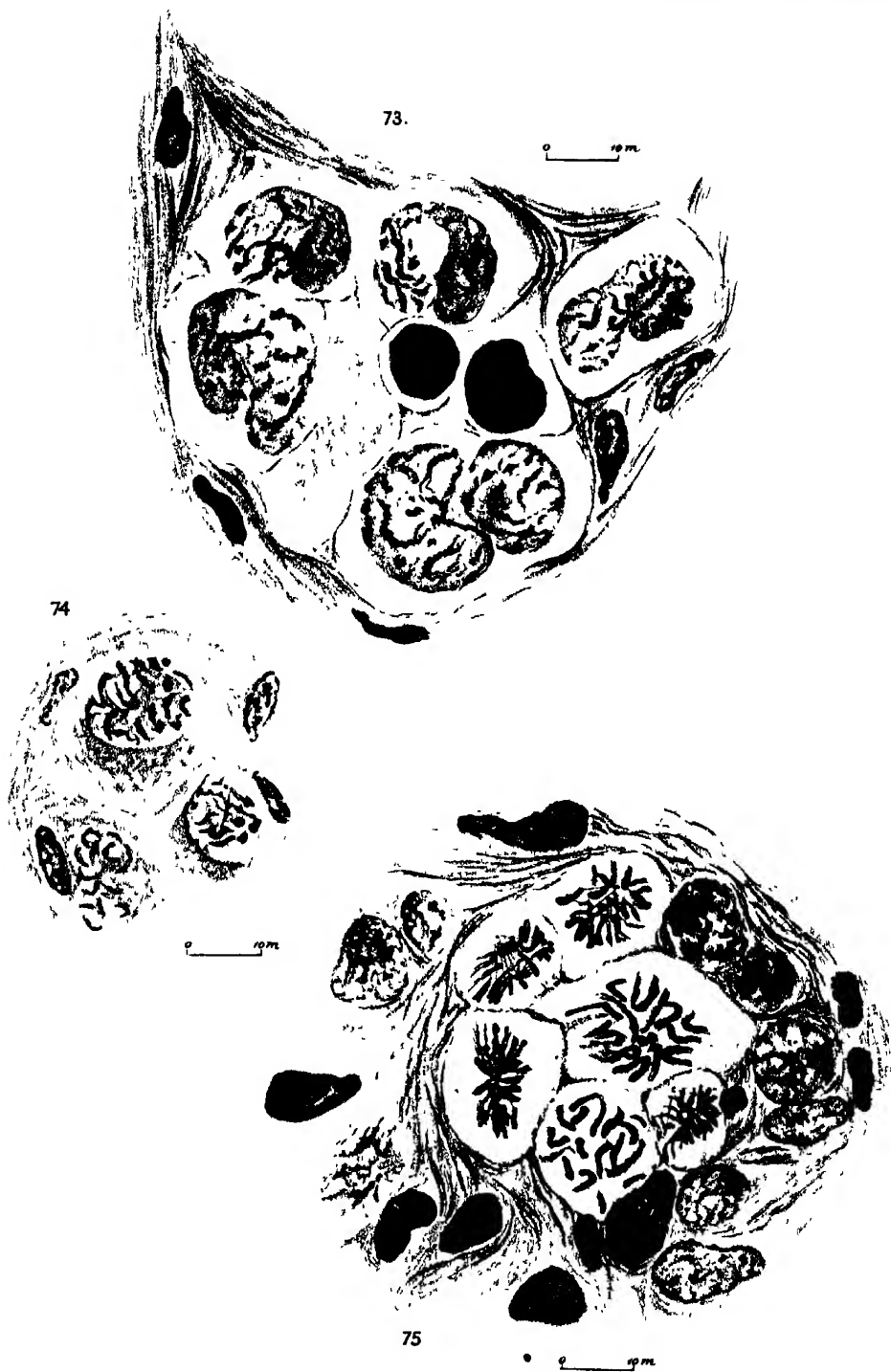


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0 10  $\mu$









- FIG. 46.—Meiotic gemini formed before disappearance of nuclear membrane.
- FIGS. 47–50.—End of prophase, metaphase, anaphase, early telophase of first Meiotic division. The splitting of the daughter chromosomes is marked in figs. 49–50.
- FIGS. 51–55.—Telophase similar to that of two preceding divisions.
- FIG. 56.—End of telophase of first and beginning of prophase of second Meiotic division.
- FIGS. 57–61.—Stages of prophase of second Meiotic division. Similar to somatic prophase. Archoplasmic vesicles shown in figs. 57 and 59 at *a*.
- FIG. 62.—Polar view of chromosomes on spindle.
- FIGS. 63 and 64.—Metaphase and anaphase of second Meiotic division. Archoplasmic vesicle at *a*, fig. 64.
- FIG. 65.—Beginning of telophase. Archoplasmic vesicles shown at *a*. Daughter chromosomes splitting into semivalent threads.
- FIG. 66.—Archoplasmic vesicles coalesce into one large vesicle *a*. Further splitting of filament.
- FIG. 67.—Archoplasmic vesicle increased in size. A darkly stained granule is surrounded by a faintly staining area, which in turn is surrounded by a clear area.
- FIGS. 68 and 69.—Archoplasmic vesicle growing and enveloping nucleus of spermatid.
- FIG. 70.—Early spermatogonia suggesting amitosis. In some nuclei the threads are clear. Others are in the stage shown in fig. 9.
- FIG. 71.—Nucleus in which the semivalent threads are approximative.
- FIG. 72.—Later stage of same.
- FIG. 73.—Same among a group of cells.
- FIG. 74.—Nuclei in spreme stage.
- FIG. 75.—Nuclei in process of Mitotic division
-

# Cell Inclusions in the Oogenesis of Scorpions.

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(Communicated by Prof J Stanley Gardiner, F.R S —Received November 11,  
1924.)

(From the Zoological Laboratory, Cambridge)

[PLATES 10-13.]

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## I INTRODUCTION.

Although the very remarkable mitochondria of the male germ cells of scorpions have attracted in the past a number of workers (12 and 13), no work had so far been undertaken on the female germ cells

In the present work two different groups of scorpions, with and without ordinary yolk in their oocytes, were selected for study in the hope that some light might be thrown on the relationship between the nucleus and yolk formation. *Euscorpius napol* and *Buthus jularcus* were selected as the representatives of those forms whose oocytes contain ordinary yolk, and *Palamnæus fulvipes madraspatensis* and *Palamnæus swammerdami* as the representatives of forms without any such yolk.

The results of investigations proved to be fruitful, in as much as they revealed a remarkable difference in the behaviour of the oocyte nucleolus in the two groups. In both *Euscorpis* and *Buthus* the nucleolus emits into the cytoplasm prominent, deeply-basophil, round bodies, whereas in *Palamnæus* it remains quite inactive.

The mitochondria and the structure, fragmentation and the ultimate transformation of the Golgi rods into the Golgi yolk have been worked out in detail in all the three genera. The oocytes of *Euscorpis* were centrifuged, and the nature of the various cell inclusions has been thus conclusively settled.

*Euscorpis* and *Buthus* resemble each other in their oogenesis in every detail, except that the fully-developed oocytes of the latter are slightly larger than those of the former. To avoid unnecessary repetition, therefore, both these forms have been treated together in one section and the figures have been selected from both the forms. All references to the figures of *Buthus* have been specified as such, and where they are not specified they belong to *Euscorpis*. The oogenesis of *Palamnæus* has been treated in a separate section.

Throughout the paper wherever the term yolk is used it means the ordinary yolk, which is proteid in nature, and is therefore not destroyed by fat solvents, like acetic acid, etc. All other kinds of yolk are specified.

I am deeply indebted to Mr. J. Gray, M.A., Fellow of King's College, under whose supervision this work has been carried out, for his extreme kindness, which has been a constant source of encouragement to me in the course of this work, and also for reading and correcting the manuscript of this paper.

## II. MATERIAL AND METHODS.

Most of the material of *Palamnæus* was brought over personally from Madras (India), and later supplies of fixed material were sent by Prof. R. Manon, of the Presidency College, and Dr. F. H. Gravely, of the Government Museum, to whom I have the great pleasure of expressing my thanks.

Large supplies of live specimens of *Buthus judarcus* and *Euscorpis napolæ* were sent by Mr. Oskar Theodor, of the Malaria Research Department, Haifa (Palestine), and Prof. F. S. Monticelli, of the Zoological Institute of the Royal University at Naples, respectively, to whom I am indebted for their very kind help. Some of the material of *Euscorpis napolæ* was personally brought over from Naples, where Prof. F. S. Monticelli very kindly arranged for all facilities in the way of collection and reagents.

As constant references will have to be made in the text to the methods used, it seems unnecessary to give here a list of the fixatives and stains employed.

### III. OOGENESIS OF *EUSCORPIUS NAPOLI* AND *BUTHUS JUDAICUS*.

#### 1. *Nucleolus and Nucleolar Extrusions.*

Plate 10, fig 1 (Bouin) shows an oocyte immediately after differentiation from the germinal epithelium. The nucleolus is a small round structure, and stains deeply and uniformly with hæmatoxylin. Long before the oocyte has pushed through the wall of the ovarian tube, the nucleolus shows signs of activity. It buds off deeply-basophil and round bodies in the nucleus. Fig 2 (Mann-Kopsch) shows two such bodies lying near the nucleolus. In fig. 3 (Champy-Kull) one such body has travelled into the cytoplasm and lies amongst the mitochondrial mass.

As growth of the oocyte continues, the nucleolus increases considerably in size and buds off nucleolar extrusions in very large numbers (figs. 4 and 5). A study of these figures clearly shows that the extrusions travel into the cytoplasm as whole bodies through temporary imperfections in the nuclear membrane.

The extrusions stain deeply with hæmatoxylin, both after Bouin and Flemming-without-acetic. If the process of differentiation in iron-alum is prolonged, it is seen that the extrusions are still black, whereas the cytoplasm has given up all stain. In Plate 13, fig. 12 (which is a toned and safranin-stained Da Fano preparation of *Buthus judaicus*), the extrusions are at N.E. and stain bright red with safranin. They stain green with thionin (Champy-Kull method), at least when they are newly formed. All these reactions show that the extrusions are made of chromatin, or something very much allied to it. Too much reliance, however, should not be placed on staining, as recent work has shown that a basophil substance is not necessarily chromatin.

In many cases, and with all the fixatives used, the extrusions were observed to lie in vacuoles. It is possible that these vacuoles are artificially formed by the sudden coagulation of the extrusions by the fixatives. But, since vacuoles are observed in many sections of all the different preparations (Bouin, Champy-Kull, Mann-Kopsch and Da Fano), they might indicate some activity on the part of the extrusions.

As a rule, the extrusions disappear when the yolk granules become prominent, although there is always some overlapping between the initial stages of yolk-formation and the last phase of nucleolar budding.

When first formed the extrusions are deeply basophil; gradually, however, they become acidophil without passing through any amphophil stage, and ultimately disappear as whole bodies, without, so far as can be ascertained, undergoing any fragmentation.

The nucleolus itself is first deeply basophil and stains uniformly, later it becomes amphophil (fig 12, *Buthus judaicus*), with an acidophil ground substance in which are embedded deeply basophil, round bodies which become the extrusions. When the process of budding has ceased and the oocyte has enormously increased in size, the basophil bodies in the nucleolus tend to disappear.

A very remarkable, although not unique, phenomenon has been observed with regard to the nucleolus both in *Euscorpis* and *Buthus*. In a number of cases (I have only about two dozen such oocytes in my possession) it has been observed that the entire nucleolus leaves the nucleus and lies in the cytoplasm, where it buds off the extrusions (fig. 7 and fig 11, *Buthus judaicus*). Such cases do not result from any rupture of the nuclear membrane due to mechanical causes; indeed there is nothing at the stage represented in figs. 7 and 11 which may cause the rupture of the nuclear membrane, for yolk puts in its appearance long after this stage.

The passage of the entire nucleolus into the cytoplasm has been described in vertebrates by Henneguy (8), who believes that the corpuscles of Balbiani are either parts of the nucleolus or the entire nucleolus, which passes through the nuclear wall into the cytoplasm.

## 2. Mitochondria.

All the usual mitochondrial fixatives and stains failed to reveal any mitochondria in the germinal epithelial cells. Soon after the oocyte has differentiated from the germinal epithelium a ring of granular mitochondria appears all round the nucleus (fig. 3, Champy-Kull). By the time the oocyte has reached the stage illustrated in fig. 4 the mitochondria have spread out uniformly throughout the oocyte. They remain granular throughout the later stages of oogenesis and hardly undergo any growth.

The mitochondria stain black with Flemming-without-acetic followed by iron-haematoxylin, violet with Benda, and pink with Champy-Kull. In the Mann-Kopsch unstained preparations they appear brownish (figs. 2 and 9). In untuned Da Fano preparations they have a beautiful golden colour (fig. 13, *Buthus judaicus*); in toned preparations they are grey. If Da Fano's method

is followed by safranin after toning, they tend to stain red (figs. 11 and 12, *Buthus judaicus*)

### 3 Golgi Apparatus, Golgi Yolk, and Ordinary Yolk.

Both the Mann-Kopsch and the Da Fano methods failed to reveal any Golgi apparatus in the germinal epithelial cells. Soon after the oocyte has been differentiated from the epithelium, however, the apparatus appears in the form of a few clearly defined curved rods lying on one side of the nucleus (fig 2, Mann-Kopsch). I have been unable to observe either any archoplasm or centrosome associated with the Golgi apparatus. As the oocyte grows the rods multiply very rapidly and are scattered throughout the cytoplasm of the oocyte (fig 9, Mann-Kopsch). At the same time, each rod breaks up into a number of grains lying one behind the other, so that the whole rod assumes a somewhat moniliform appearance (fig. 9, G.A.). It is in untuned Da Fano preparations, however, that the segmentation of the rods is most clearly studied. This is due to the fact that the beautiful golden mitochondria form a very favourable background for the study of the black Golgi rods. In fig. 13 (which is an untuned Da Fano preparation of *Buthus judaicus*) the small granules at M. are the mitochondria, and the Golgi rods appear as straight, curved, U-shaped, V-shaped, and X-shaped rods. The last appearance, of course, results from two rods crossing each other. Almost all the rods are breaking up into granules lying one behind the other. At G.y. are the black granules which have resulted from the breaking up of the rods. These granules grow in size and give rise to the Golgi yolk (figs. 10 and 14, G.y.).

The Golgi yolk is blackened intensely like the Golgi rods, both by the Mann-Kopsch and Da Fano methods. If the Mann-Kopsch preparations are kept in turpentine, the Golgi yolk resists decolorisation for many hours, like the Golgi rods. In many cases such preparations were left in turpentine overnight and in the morning both Golgi yolk and Golgi rods were still black. Again, both in toned and untuned Da Fano preparations both Golgi yolk and Golgi rods appear black. Lastly, in chrome-osmium preparations neither the Golgi yolk nor the Golgi rods appear.

All these reactions very clearly show that the Golgi yolk is similar to the Golgi rods in its chemical constitution and does not contain any free fat. If it had contained any free fat, it could not possibly resist decolorisation by turpentine for such a long time, and it would have appeared black in chrome-osmium. Again, it would not have gone black in Da Fano preparations. All

these important reactions will become still more clear when centrifuged oocytes are studied in a later section.

The ordinary yolk puts in its appearance a very long time after the appearance of the Golgi yolk. The latter appears at the very beginning of the growth of the oocyte (fig. 11; G.y.). The ordinary yolk, on the other hand, appears first as very small granules some time when the process of nucleolar budding is about to cease (fig. 14; D y.).

The ordinary yolk is very slightly blackened, if at all, both by the Mann-Kopsch and the chrome-osmium methods; but, unlike the Golgi yolk, it can be decolorized in turpentine in less than five minutes. In untuned Da Fano preparations the ordinary yolk is somewhat grey and slightly tinged golden, and cannot be distinguished from the bright golden mitochondria except in centrifuged oocytes. In toned sections the ordinary yolk is grey like the mitochondria.

#### 4 *Perinuclear Region*

Some time after the process of nucleolar budding has well advanced and after the oocyte has pushed through the wall of the ovarian tube and attained a fairly large size, a ring of dense cytoplasm, not to be confused with the mitochondrial ring of very young oocytes, appears all round the nucleus. I have spoken of this ring as the perinuclear region of the cytoplasm.

In Bouin preparations (fig. 6, P.N.R.) the perinuclear region consists of cytoplasmic granules much more densely packed than in the peripheral cytoplasm. During the process of differentiation of the hæmatoxylin stain in iron-alum, this region gives up stain rather later than the peripheral cytoplasm, not because the granules of the ring are in any way more chromatic but because they are densely packed.

The perinuclear region appears very black in Mann-Kopsch preparations (fig. 9). In this figure it has been cut obliquely and consists of Golgi grains so intensely blackened with osmic acid that it resists decolorization in turpentine for days. In fact, I have found it impossible to decolorize this region, and in many cases it is so uniformly blackened that no structure can be made out in it.

With chrome-osmium followed by the usual mitochondrial stains the perinuclear region is seen to consist of mitochondria more densely packed than in the peripheral cytoplasm. In other words the perinuclear region consists of cytoplasmic granules, mitochondria and Golgi rods more densely packed than in the peripheral cytoplasm. It disappears when the process of nucleolar budding is over and the yolk granules become prominent.



### 5. *Effects of Centrifugal Force on Various Cell Inclusions.*

Fresh ovaries are centrifuged with a hand centrifuge for about half an hour in normal salt solution.

Fig. 14 shows a centrifuged oocyte, in which the process of yolk formation has not very much advanced, fixed with Mann-Kopsch. The yolk granules at D.y. are small and are thrown down, and appear yellowish. The Golgi yolk at G.y. is intensely black and is at the opposite pole. This area may sometimes be so intensely and uniformly blackened by osmic acid that it is impossible to decolorize it in turpentine. The central area appears brownish and consists of granular mitochondria with a large number of irregularly scattered Golgi rods.

With Da Fano most beautiful preparations result. The ordinary yolk appears somewhat grey and the Golgi yolk appears intensely black, as in the Mann-Kopsch preparations. The central area is vacuolated and contains beautiful golden mitochondria and the black Golgi rods.

While there is always a very sharp line of demarcation between the ordinary yolk area and the mitochondrial area, the Golgi yolk area passes more or less gradually into the mitochondrial area—the latter being never free from the Golgi grains which are not yet converted into the lighter Golgi yolk. With the further growth of the oocyte and the consequent enlargement of the ordinary yolk bodies, the Golgi yolk does not come up in the centrifuged egg. In *Lithobius*, however, the Golgi yolk comes up even in the most highly developed oocyte, because it contains free fat and is therefore very light.

Paucity of suitable material has not enabled me to fix centrifuged oocytes with chrome-osmium, but from the study of the uncentrifuged oocytes it may quite safely be inferred that neither the Golgi yolk area nor the Golgi rods and grains of the mitochondrial area will appear in such preparations.

Amongst the minor effects of the centrifugal force may be mentioned the disappearance of the denser perinuclear region

### IV. OOGENESIS OF *PALAMNÆUS FULVIPES MADRASPATENSIS*.

The oocytes of *Palamnæus* have no ordinary yolk, and the mode of development of the embryo is unique, inasmuch as it takes place first at the expense of the inner layer of the diverticulum in which the embryo lies, and later at the expense of a cord of cells (which belong to the outer layer of the diverticulum), forming a coiled appendix whose lower end lies just opposite the opening of the stomodœum of the embryo. Correlated with this specialized mode of

nutrition the fully developed oocyte of *Palamnæus* is a much smaller structure ( $0.0615 \times 0.0384$  mm.) than that of either *Euscorpius* or *Buthus*, whose oocytes are comparatively enormous (*Euscorpius*  $0.4 \times 0.2$  mm.), being packed with large yolk discs

From a study of figs. 15 and 16 (Bouin) it will be clear that there is no ordinary yolk. The cytoplasm consists of very prominent and coarse strands, forming alveoli at certain places and irregular networks at others.

When Mann-Kopsch preparations are mounted unstained, the whole cytoplasm is intensely and almost uniformly blackened and hardly reveals any structure. When the slide is kept in turpentine for at least one hour, an appearance like that of fig. 19 results. At G.A. are small Golgi rods which appear very black. At G.y is the Golgi yolk, consisting of black, very prominent bodies of different sizes. The reasons for regarding these bodies as the Golgi yolk will be given when the results of the various fixatives have been described. At M. are small granular mitochondria, which appear brownish. The comparatively small oocyte is so packed with these inclusions that the cytoplasmic strands shown in figs. 15 and 16 are not visible.

The slide is then left in turpentine overnight. In the morning the appearance shown in fig. 20 results. At G.A. are the Golgi rods, which are still black. At M. are the brownish granular mitochondria. Almost the whole of the Golgi yolk has disappeared, except a few granules marked Y.G.y, which still remain black, and which I consider to be young Golgi yolk, having the same chemical constitution as the Golgi rods and as yet not having undergone any fatty degeneration like the fully developed Golgi yolk bodies, which have entirely disappeared. In place of the Golgi yolk bodies are seen certain alveoli, which I believe to be cytoplasmic (cf. figs. 15 and 16). The alveolar area (C.T.) appears quite white in sharp contrast to the black Golgi rods and the brownish mitochondria

In fig. 17 (chrome-osmium and iron-hæmatoxylin) at G.y is the Golgi yolk, which is blackened intensely by chrome-osmium in ten hours, as unstained sections clearly show. The Golgi rods, of course, do not appear in this preparation. At M. are the granular mitochondria, which are stained black, with iron-hæmatoxylin.

If an unstained chrome-osmium preparation is left in turpentine overnight, the Golgi yolk bodies completely disappear, and in the morning no young Golgi yolk granules appear, unlike the Mann-Kopsch preparations.

In Bouin's preparations (figs. 15 and 16) no cell inclusions whatsoever appear. In Flemming-with-acetic acid, however, the Golgi yolk does appear and is very

black in unstained slides (fig. 18). This seems to be due to the fact that the solvent action of the acetic acid is hindered by osmic acid.

Lastly, sections of the embryos of two-layered stage fixed with Mann-Kopsch show that the Golgi yolk has entirely disappeared. It is, therefore, clear that the Golgi yolk has a nutritive function in the early segmentation stages.

It remains now to be established that the bodies which I have described as the Golgi yolk are derived from the Golgi rods. I admit that the evidence on this point is not so strong as in the case of *Euscorpius* and *Buthus*, and this is undoubtedly due to the small size of the oocyte and the consequent overcrowding of the various cell inclusions. Nevertheless whatever evidence is available, considered in conjunction with the cases of other scorpions, has convinced me that the Golgi yolk is derived from the Golgi rods by a process of fatty degeneration, similar to that of *Lithobius* (11) described by me.

When Mann-Kopsch preparations are kept in turpentine, and the slide studied at intervals, it is noticed that the Golgi yolk bodies are of different sizes, and that they are not decolorised at the same rate. Some are decolorised quickly, while others take many hours to decolorise, and there are some which are not decolorised even after one night's immersion (fig. 20; Y.G y.). This clearly shows that there are intergradations between the Golgi rods proper and the fully developed Golgi yolk which contains free fat.

## V. DISCUSSION.

After having studied the various cell inclusions in the two forms of scorpions with and without ordinary yolk in their oocytes, and with remarkably different modes of nutrition, we are now in a position to discuss very briefly certain problems relating to their origin and function. These problems will be discussed under the following heads :—

### 1. Nucleolar Extrusions and the Ordinary Yolk.

We have seen that the fully developed oocytes of *Euscorpius* and *Buthus* are full of large discs of ordinary yolk, whereas those of *Palamnæus* have no such yolk. Again, in both *Euscorpius* and *Buthus* there is a copious discharge of prominent, round and deeply-basophil bodies from the nucleolus into the cytoplasm. In *Palamnæus*, on the other hand, the nucleolus remains quite inactive, and does not discharge any such bodies. These facts certainly suggest some relationship between nucleolar extrusions and yolk formation.

The nucleolar extrusions are much larger than the young yolk granules (fig. 14) and the former never undergo any fragmentation. They are first basophil, and later, becoming acidophil, disappear as whole bodies. It may therefore be said, with a fair amount of certainty, that the extrusions are not directly transformed into yolk granules.

On the other hand, it is quite possible that when the extrusions disappear, their substance might contribute towards the formation of the yolk.

The cases of *Lithobius* (Nath, 11) and *Saccocirrus* (Gatenby, 5) are somewhat different from those of the scorpions. In these forms the nucleolar extrusions undergo fragmentation, and give rise to small granules which may be directly transformed into yolk granules. Prof. Gatenby thinks that such a process takes place in *Saccocirrus*, but, so far as my work on *Lithobius* is concerned, there is no direct evidence to show that the extrusions are directly transformed into yolk granules, although I feel certain that the extrusions indirectly contribute towards the formation of yolk.

That there is some relationship between yolk and nuclear or nucleolar extrusions is also suggested by the fact that, to the best of my knowledge, there is no clearly established case of such extrusions in Metazoan oocytes in which yolk is not ultimately developed.

## 2. Golgi Yolk.

We have seen that in all the three genera of scorpions the Golgi rods swell up after fragmentation, and give rise to yolk bodies obviously having a nutritive function. In *Euscorpius* and *Buthus* the Golgi yolk retains the same chemical constitution as the Golgi rods, whereas in *Palamnæus* it contains free fat, like that of *Lithobius* (Nath, 11) and *Helix aspersa* (Brambell, 2), inasmuch as it is blackened by chrome-osmium alone.

The only other cases in the whole animal kingdom, so far as I know, in which the Golgi elements are directly converted into yolk are *Saccocirrus* (Gatenby, 5) and *Ascidians* (Hirschler, 9). In *Patella* (Ludford, 10, Gatenby and Woodger, 7, and Brambell, 2), however, the Golgi elements participate indirectly in the process of Vitellogenesis. In many places the Golgi elements collect together and the cytoplasm between them becomes more stainable under their influence. Gradually this stainable cytoplasm swells up into a yolk sphere with the Golgi elements stuck to its surface and almost completely surrounding it.

### 3. Perinuclear region.

The perinuclear region is simply a denser region of the cytoplasm with mitochondria and Golgi elements more densely packed than in the peripheral cytoplasm. It appears when the egg is about 0.154 mm. long and 0.123 mm. broad and always disappears before yolk puts in its appearance. It should not be confused with the so-called "yolk nucleus" of invertebrates (*e.g.*, *Antedon* (Chubb, 4), *Lumbricus*\* (Calkins, 3), and *Pholcus* (Bambecke, 1), etc., etc., and vertebrates (Henneguy, 8), which is of nuclear origin, in some cases real and in others supposed, and which exists in the cytoplasm either as a single compact body or in the form of scattered granules, and is supposed to control either directly or indirectly the formation of yolk. Obviously, the nucleolar extrusions of *Euscorpis* and *Buthus* correspond to the so-called "yolk nucleus" of the forms described above.

The exact significance of the perinuclear region I am unable to point out; nor do I know of a similar structure described for any other egg. It need hardly be added that the remarkable denseness of the cytoplasm immediately round the nucleus probably shows that the nucleus is exerting some influence on the cytoplasm.

### 4 Mitochondria.

The mitochondria in all the three genera of scorpions studied are granular, whereas in the male germ cells they are very prominent, vesicular bodies. The remarkable mitochondria of the male germ cells of *Palamnæus* and other scorpions led me to regard the oily yolk of the oocyte of *Palamnæus* as mitochondria, but a comparative study of the mitochondria in the oogenesis of other scorpions soon showed that they are really granular in all these forms.

The only other case of such remarkable difference in the size of the mitochondria of the male and the female germ cells is, so far as I know, that of *Paludina vivipara* (Gatenby, 6). Even in this form, it is only the cells forming the "typic" spermatozoa, which have a few large, stout, rod-like mitochondria, in sharp contrast to the granular ones of the egg; whereas the cells forming the "atypic" spermatozoa have mitochondria similar to those of the egg.

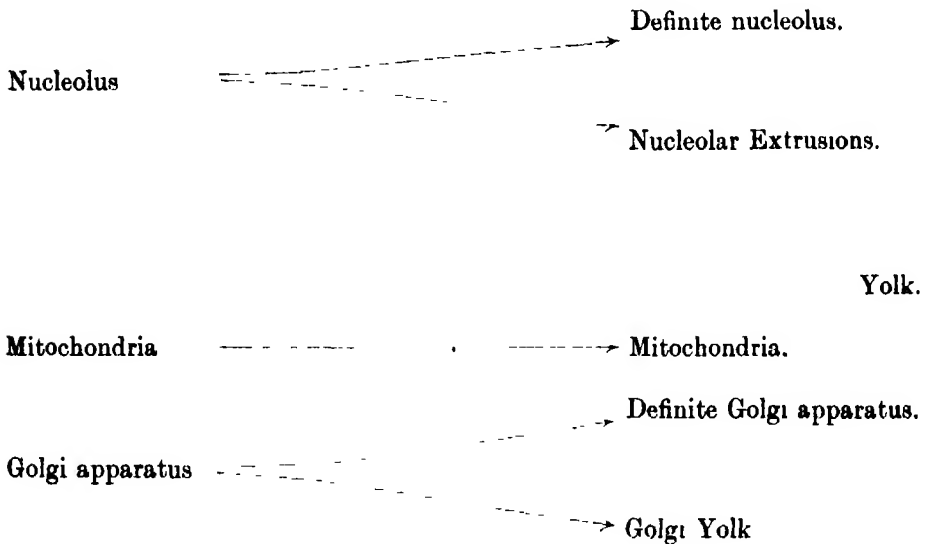
This marked difference in the size of the mitochondria in the two sexes of scorpions becomes less remarkable, however, when it is remembered that in

\* In a forthcoming paper I have shown that the so-called "yolk nucleus" of *Lumbricus* is really the remarkable Golgi apparatus.

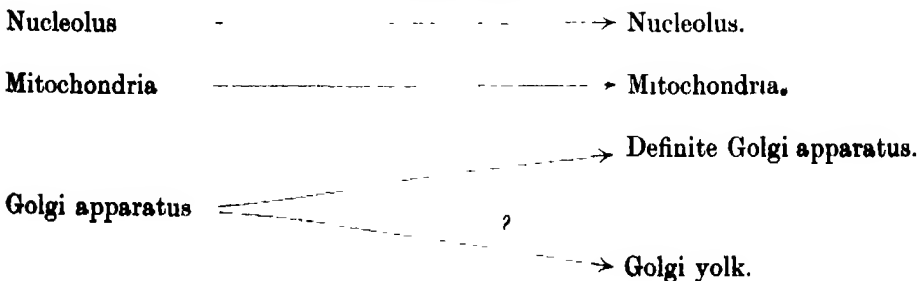
*Palamncæus*, which I have recently investigated, the mitochondria arise as more or less granular bodies in the first spermatogonial generation. They enlarge into very prominent, vesicular bodies in the growth stage and during spermateleosis they unite to form a mitosome. This disintegrates into fragments of mitochondrial material which descend down the axial filament and becoming smaller and smaller ultimately disappear.\* So that even in the male germ cells the mitochondria may be said to be granular, the vesicular type being only a phase in spermatogenesis.

The various cell inclusions in the oogenesis of scorpions may be graphically represented thus :—

*Euscorpilus and Buthus.*



*Palamncæus.*



\* In this connection, however, see a forthcoming paper in 'Quart. Jour. Micr. Sci.'

## VI. SUMMARY.

1. In both *Euscorpius napolé* and *Buthus judaicus* whose oocytes contain ordinary yolk, which is not destroyed by fat solvents like acetic acid, the nucleolus increases considerably in size and emits into the cytoplasm deeply basophil, round bodies. On the other hand, in *Palamnæus fulvipes madras-patensis*, whose oocytes have no such yolk, the nucleolus remains quite inactive.

2. The nucleolar extrusions are at first basophil and later, becoming acidophil, disappear as whole bodies. Although there is an overlapping between the initial stages of yolk formation and the last phase of nucleolar budding, the extrusions disappear when the yolk granules become prominent.

3. Evidence has been adduced that the extrusions are not directly transformed into yolk granules. Indirect evidence already furnished strongly suggests that there is some relationship between nucleolar budding and yolk formation. The exact nature of this relationship it is impossible to ascertain.

4. The structure, fragmentation and the ultimate transformation of the Golgi rods into the Golgi yolk has been worked out in three genera of scorpions.

5. In both *Euscorpius* and *Buthus* the Golgi yolk retains the same chemical constitution as the Golgi rods and does not contain any free fat.

6. In *Palamnæus* the oily yolk which probably arises from the Golgi rods contains free fat, like the Golgi yolk of *Lithobius* (Nath, 11) and *Helix aspersa* (Brambell, 2)

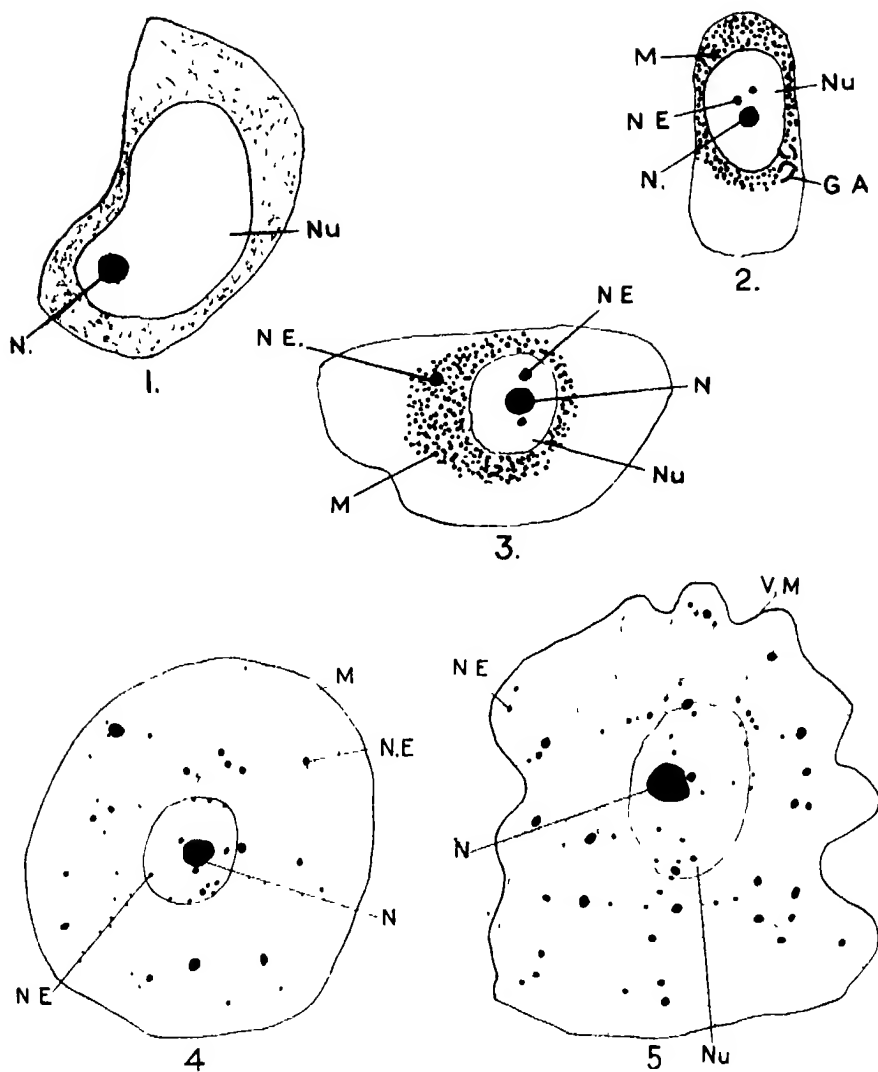
7. The mitochondria in the oocytes of all the forms studied are granular like those of most other eggs, but in the male germ cells they are very prominent, hollow, vesicular bodies. Such a marked difference in the size of the mitochondria of the male and the female germ cells has been described, so far as I know, in one other form only, namely, *Paludina vivipara* (Gatenby, 6).

8. In the centrifuged oocytes of *Euscorpius* the ordinary yolk is thrown down and the Golgi yolk forms the uppermost layer. The mitochondria and numerous irregularly scattered Golgi grains form the central area. The nucleus lies in the centre of this area.

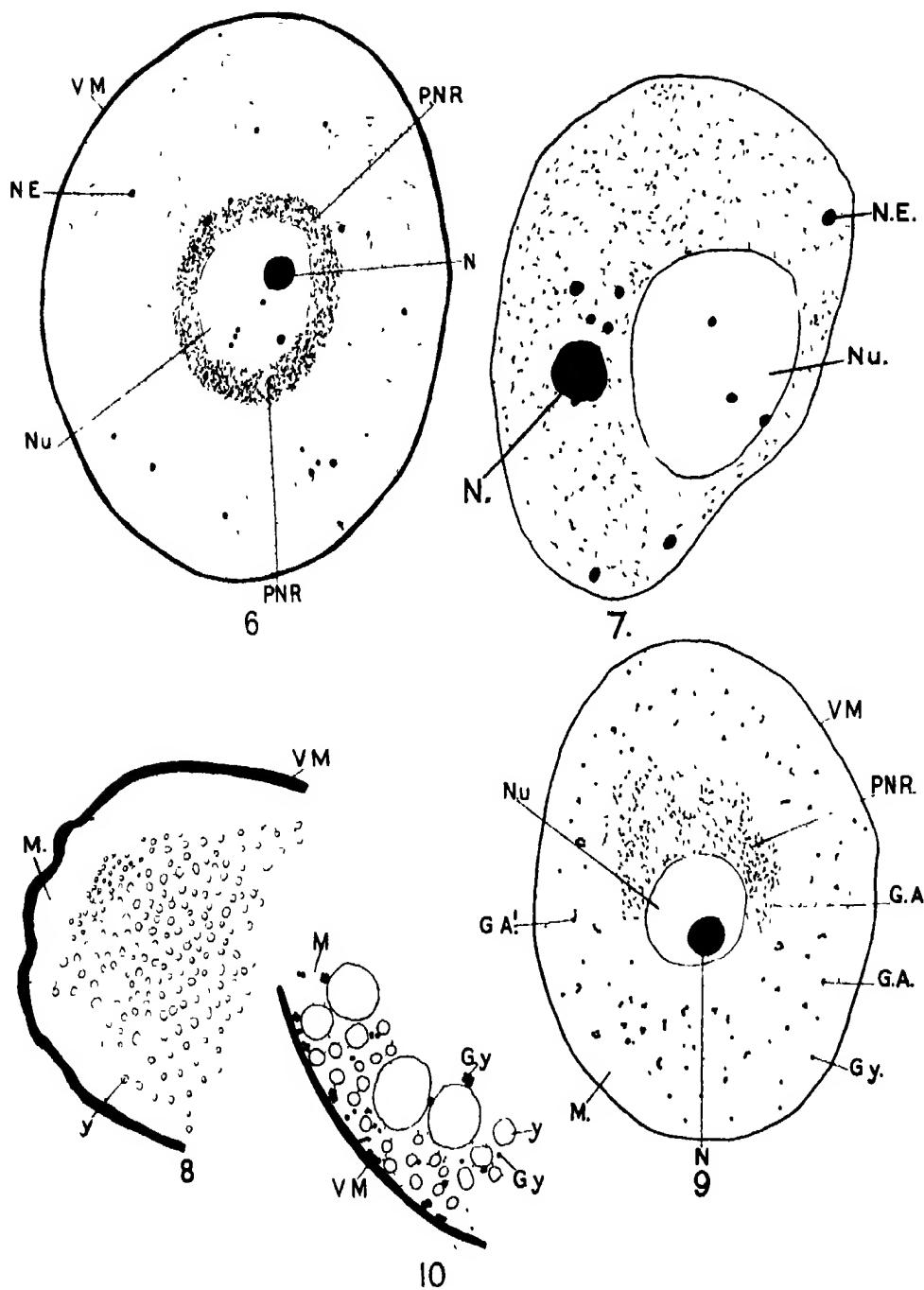
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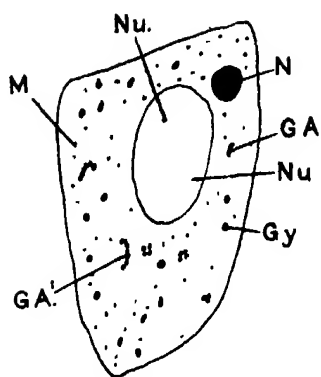
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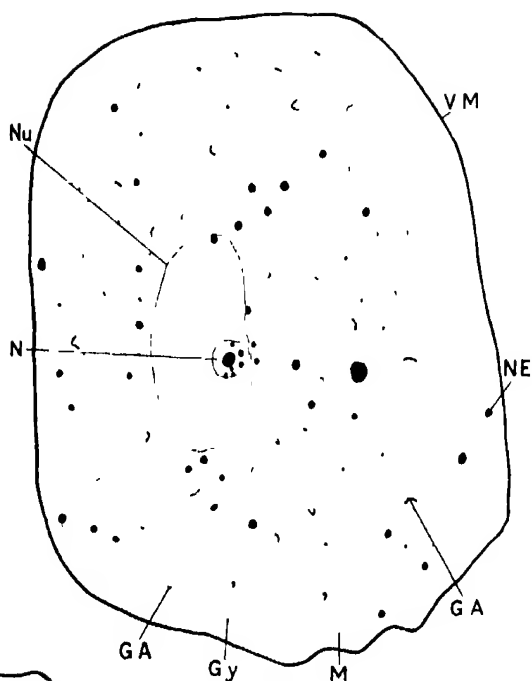




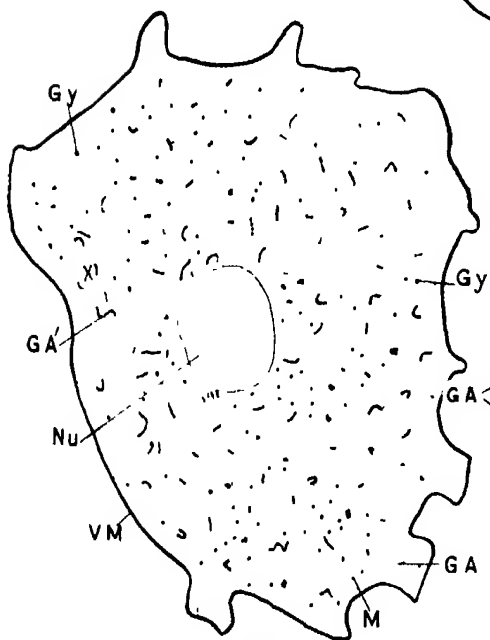




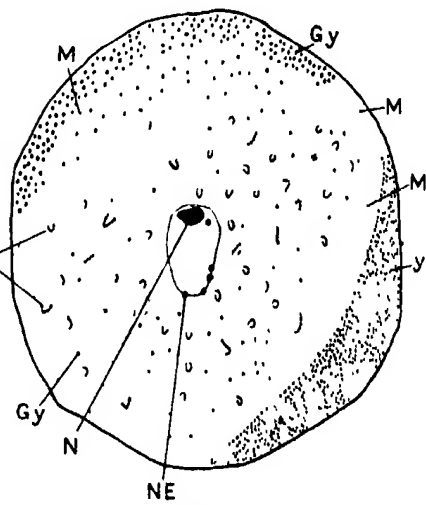
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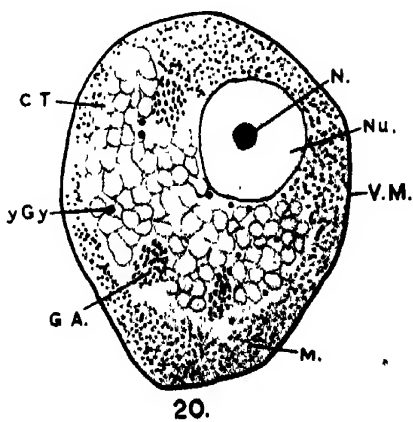
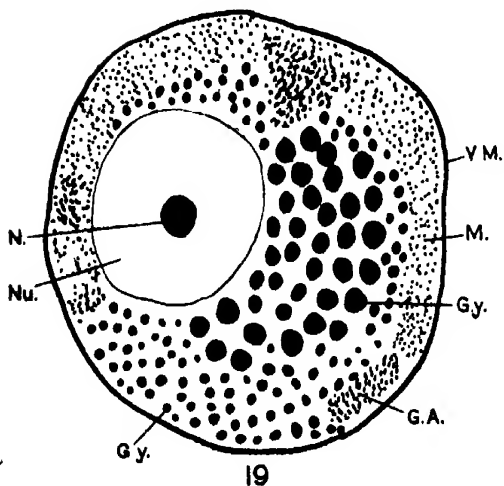
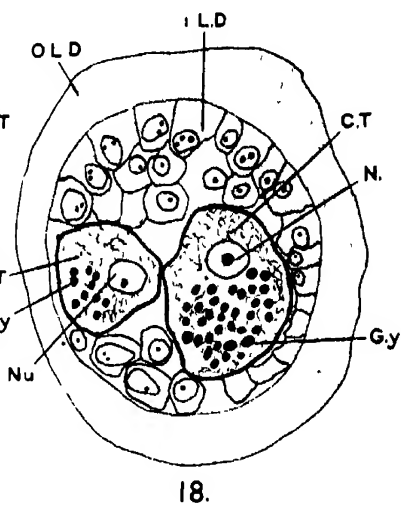
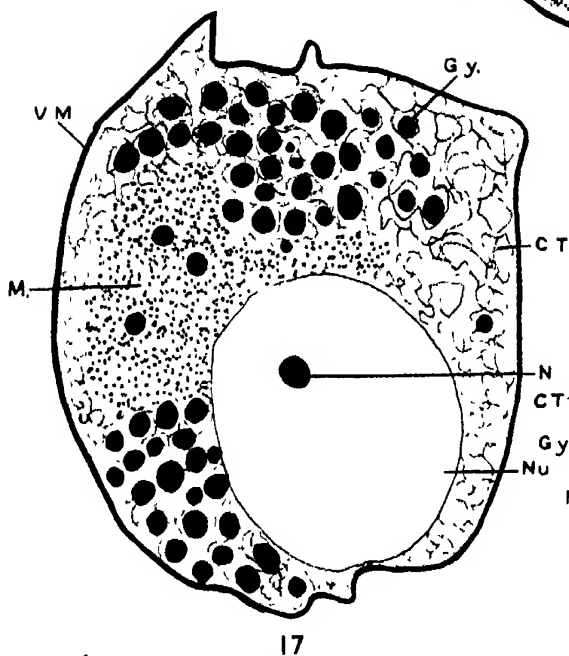
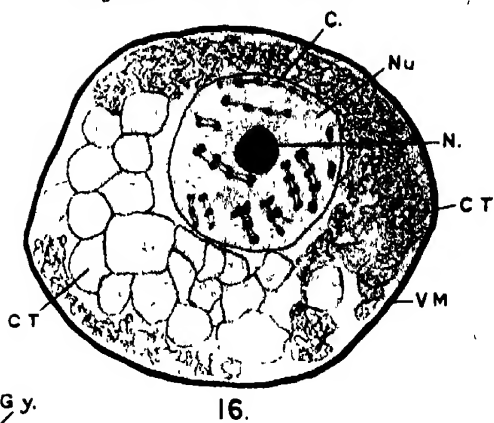
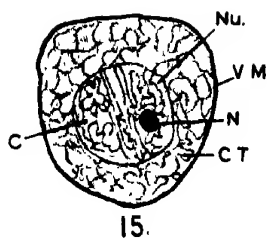
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# VIII.—EXPLANATION OF PLATES 10-13

FIGS. 1 to 10 and 14.—*Euscorpis napol.*

FIGS. 11, 12, and 13.—*Buthus judaicus.*

FIGS. 15 to 20.—*Palamnaeus fulvipes madraspatensis*

## REFERENCE LETTERS.

C. = chromosomes. Ch. = chorion. Cy.G. = cytoplasmic granules C.T. = cytoplasmic strands G.A. = Golgi rods G.A.' = Golgi rod fragmenting G.y. = Golgi yolk. I.L.D. = inner layer of diverticulum. M. = mitochondria. N. = nucleolus. Nu. = nucleus N.E. = nucleolar extrusions. O.L.D. = outer layer of diverticulum. P.N.R. = perinuclear region V.M. = vitelline membrane. D.y. = yolk Y.G.y. = young Golgi yolk.

## PLATE 10.

- FIG. 1.—Showing an oocyte just after differentiation from the germinal epithelium. Nucleolar budding has not yet started. Bouin × 1560.
- FIG. 2.—An oocyte of about the same stage as shown in Fig. 1, showing Golgi rods, mitochondrial ring, and beginning of nucleolar budding. Mann-Kopsch. × 660.
- FIG. 3.—A very young oocyte, showing nucleolar budding and mitochondrial ring. Champy-Kull. × 660.
- FIG. 4.—Showing nucleolar budding and mitochondria. Nucleolar extrusions constructed from nine consecutive sections F. W. A. and iron hæmatoxylin × 330.
- FIG. 5.—Showing nucleolar budding. Nucleolar extrusions constructed from three consecutive sections Bouin. 330

## PLATE 11.

- FIG. 6.—Showing nucleolar budding and dense perinuclear region Bouin about 300.
- FIG. 7.—Showing the passage of the entire nucleolus into the cytoplasm. Nucleolar extrusions constructed from five consecutive sections Bouin. about 660.
- FIG. 8.—A portion of a well-advanced oocyte, showing mitochondria and yolk Champy-Kull. about 300.
- FIG. 9.—Showing Golgi rods, Golgi yolk, mitochondria and perinuclear region cut obliquely which shows densely packed Golgi rods. Mann-Kopsch about 330.
- FIG. 10.—A portion of the most highly developed oocyte showing large yolk discs, mitochondria and Golgi yolk. Mann-Kopsch Semi-diagrammatic

## PLATE 12

- FIG. 11.—A very young oocyte, showing the passage of the entire nucleolus into the cytoplasm, mitochondria and Golgi rods. Da Fano, toned and safranin stained. × 660.
- FIG. 12.—Showing nucleolar extrusions, Golgi rods, Golgi yolk, and mitochondria. Nucleolar extrusions constructed from nine consecutive sections. Da Fano, toned and safranin stained. about 320.

FIG. 13.—Showing the fragmentation of the Golgi rods into Golgi yolk, and the mitochondria. Da Fano, untuned.  $\times$  about 320.

FIG. 14.—Centrifuged oocyte, showing three areas. Mann-Kopsch.  $\times$  about 180. Semi-diagrammatic

PLATE 13.

FIGS 15 and 16.—Showing coarse cytoplasmic strands and the inactive nucleolus. Boum.  $\times$  about 1400.

FIG 17.—Showing granular mitochondria and very prominent Golgi yolk. F. W. A. and iron hæmatoxylin.  $\times$  about 1400.

FIG 18.—A transverse section through a diverticulum, showing two oocytes with Golgi yolk Flemming.  $\times$  about 640.

FIG. 19.—A Mann-Kopsch preparation kept in turpentine for one hour, showing granular mitochondria, Golgi rods, and very prominent Golgi yolk.  $\times$  about 1400.

FIG. 20.—A Mann-Kopsch preparation kept in turpentine overnight. Golgi yolk has completely disappeared, but Golgi rods and young Golgi yolk are still black Mitochondria granular  $\times$  about 1370

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*The Isolation of a Product of Hydrolysis of the Proteins hitherto undescribed.*

By S. B. SCHRYVER, D.Sc., Ph.D., Professor of Biochemistry, Imperial College of Science and Technology, H. W. BUSTON, B.Sc., and D. H. MUKHERJEE, M.B., B.Sc.

(Communicated by Prof. V. H. BLACKMAN, F.R.S.—Received January 5, 1925.)

Some time ago, two of the authors of the present communication, in seeking a method for the separation of the amino-acids from the carbohydrates, found that under certain conditions the former could be readily separated in the form of the barium salts of their carbamates, a class of compounds originally described by Siegfried. As these carbamates, on heating with water, are readily decomposed into barium carbonate and the free amino-acid, it was suggested that a convenient method might be evolved, using the formation of these compounds as a basis, for the separation of the hydrolysis products of the proteins.\*

This suggestion was followed up, and a method was subsequently elaborated and applied to the separation of the hydrolysis products of gelatin by one of the authors in conjunction with Miss H. L. Kingston.† Since the publication

\* Buston and Schryver, 'Biochem. J.', vol. 15, p. 636 (1921).

† Kingston and Schryver, 'Biochem. J.', vol. 18, p. 1070 (1924).

of the two papers just quoted, the researches on the use of the "carbamate method," as it may be conveniently called, have been continued, and promise results, which may ultimately lead to a satisfactory separation of most of the hydrolysis products of the proteins when only relatively small amounts of material are available for investigation. During the course of this work the base, which is the chief subject discussed in this paper, was discovered.

*Isolation of the Base  $C_6H_{14}O_3N_2$  from Isinglass.*

In the experiments on the isolation of the hydrolysis products of gelatin, the material employed was Coignet's "gold label," a product of mammalian origin. It was considered to be of interest to compare the products obtained from this gelatin with those obtained from one of widely different origin. For this purpose the gelatin from the swim-bladder of sturgeon was chosen. This was prepared in the laboratory from the dried bladder, a product known commercially as *Balouka*. This was extracted with water at 80°, and the jelly thus obtained was dried down in sheets in the usual manner. A perfectly white transparent product was thereby obtained. This was hydrolysed by throwing it in small pieces into boiling 25 per cent. sulphuric acid. This procedure was adopted in order to bring about the scission of the peptide linkages as rapidly as possible, for it has been shown that in the presence of cold acids rearrangement in the molecules take place, giving rise to products which, on hydrolysis, yield substances differing from those obtained by the rapid hydrolysis of the original gelatin.\*

After hydrolysis, the sulphuric acid was removed by barium hydroxide and the dicarboxylic acids were precipitated as barium salts in the presence of alcohol. From the filtrate from these, the other amino-acids were separated in the form of the barium salts of the carbamates, the general methods employed being those described in the paper of Kingston and Schryver (*loc. cit.*). The barium carbamates were then extracted with ice-cold water. Now in the case of the gelatin of mammalian origin, the only barium carbamate which was insoluble in cold water was that of glycine. On treating this for a prolonged period with hot water it decomposed into barium carbonate and glycine, and the latter substance was obtained immediately in a pure form on evaporation of the filtrate from the barium carbonate. In the case of casein, it was found that, with the exception of a small trace, all of the barium carbamates were soluble in ice-cold water. The insoluble barium carbamate fraction from

\* Cf. Knaggs, "Notes on the determination of the Hausmann numbers." 'Biochem. J.,' vol. 17, 488 (1923).

isinglass was in one respect markedly different from that obtained from the mammalian gelatin. The filtrate from the barium carbonate got by its decomposition with hot water, contained in addition to glycine, a basic product which could be precipitated from solution (made acid with 5 per cent. sulphuric acid) by phosphotungstic acid. As arginine, lysine and histidine have already been found to give barium carbamates readily soluble in ice-cold water, this result indicated the presence amongst the hydrolysis products of isinglass of a base hitherto undescribed.

#### *Purification of the Base.*

The phosphotungstate of the base was decomposed by barium hydroxide in the usual manner. The base was thereby obtained in the form of a yellow syrup, from which on prolonged standing small hard crystals slowly separated. The syrup was then dissolved in water so as to make a 5 to 10 per cent. solution, to which aqueous mercuric chloride and saturated barium hydroxide were added, the former in excess, and the latter until yellow mercuric oxide began to be precipitated. A pale buff-coloured precipitate is formed, which can be dissolved in a large amount of boiling water, from which a crystalline precipitate separates on cooling. Boiling the precipitate with water appears to convert the mercuric salt into a basic salt. The original buff-coloured precipitate was not usually treated with boiling water, but after washing, was suspended in cold water and decomposed by hydrogen sulphide. The filtrate from the mercuric sulphide was freed from chloride by silver sulphate, and the Ag and  $\text{SO}_4$  ions were removed afterwards in the usual manner. The solution thus obtained was concentrated *in vacuo* to a syrup, which was then converted into a granular product by treatment with absolute alcohol. It was finally dried *in vacuo* at  $100^\circ$  over phosphorus pentoxide. The substance is very hygroscopic, and can only be dried to constant weight by prolonged heating *in vacuo* at  $100^\circ$ . As it also tends to absorb carbon dioxide from the air, owing to its strongly basic character, it is necessary to exercise great care in handling it. It will be noticed, in the analyses quoted below, that the carbon is slightly higher than that required for the formula  $\text{C}_6\text{H}_{14}\text{O}_3\text{N}_2$ . This is due to the slight, almost unavoidable formation of traces of carbonate during manipulation. It was obtained by the method described in the form of a cream-coloured powder. It is intensely soluble in water, but insoluble in almost all organic solvents; it dissolves in a boiling mixture of pyridine and glacial acetic acid.

The salts are also intensely soluble in water. Repeated attempts to obtain

a crystalline picrate failed. A crystalline nitrate, separating in clusters of needles, was obtained ; this also was very deliquescent, as was also the copper salt, obtained by boiling a solution of the base with freshly-precipitated cupric hydroxide. The aurichloride forms golden-yellow needles, which can be precipitated directly from solutions in hydrochloric acid. The base cannot be precipitated as a silver salt, even in solutions made alkaline by barium hydroxide. It can thus be separated from arginine and histidine, and should, therefore, in the ordinary procedure for separating the hexone bases, be found in the lysine fraction. The analyses indicate that the base differs in empirical formula from the last-named base only in that it contains an extra oxygen atom. It is proposed to call it provisionally *oxyllysine*.

The molecular weight of such a substance should be 162.

The molecular weight of the substance obtained was determined by the estimation of the depression of the freezing point of water by Beckmann's method and found to be 155.

0.1951 g dissolved in 20 g water gave a depression of  $0.117^{\circ}$ . The molecular weight was also determined by titrating a solution of the base with sodium hydroxide in the presence of formaldehyde (Sørensen's method).

0.4368 g base required 28.0 c.c. N/10 NaOH

0.2001 g. „ „ 12.50 „ „ „

From these two determinations the molecular weights calculated 156 and 160. The nitrogen can be readily determined by Kjeldah's method. By this method 17.36, 17.36 and 17.41 per cent. was found.

( $C_6H_{14}O_3N_2$  requires 17.29 per cent.)

(i) 0.1092 g. gave 0.1798 g.  $CO_2$  and 0.0812 g.  $H_2O$

(ii) 0.1896 g. gave 0.3110 g.  $CO_2$  and 0.1428 g.  $H_2O$ .

Found C (i) 44.91, (ii) 44.74 per cent, and H (i) 8.26, (ii) 8.37 per cent.

$C_6H_{14}O_3N_2$  requires C = 44.44 and H = 8.64 per cent

(Compare note given above on difficulty of avoiding carbonate formation)

The base did not melt without decomposition ; it was furthermore optically inactive. The amino-acids tend to become racemised during the carbamate process. A sample of leucine prepared by this method was also found to be almost completely inactive.



*Action of Nitrous Acid on the Base.*

The action of nitrous acid on this base is almost exactly the same as on lysine. About two-thirds of the nitrogen are rapidly evolved, but the remaining third is evolved much more slowly. One only of several experiments carried out in the Van Slyke apparatus need be quoted, as the others gave similar results. Sixty-three per cent. of the total was evolved in 10 minutes and 92.4 per cent. in 24 hours. There is no doubt, therefore, that both the nitrogens are in the form of amino-nitrogen.

*The Preparation of the Benzoyl Compound.*

0.5 g of the base were dissolved in 2-3 c.c. of *N* NaOH solution and shaken with the equivalent of nine molecules of benzoyl chloride and sodium carbonate in the usual manner (Baumann-Schotten). After completion of the reaction excess of sulphuric acid (1 vol. water. 1 vol. concentrated acid) warmed to 60-70° was added. The benzoyl compound together with benzoic acid is thereby precipitated. The white crystals of the acid can be readily separated from the yellowish pasty benzoyl derivative by repeated washing with water at 60° C. The latter was finally dried in a vacuum desiccator. It is readily soluble in alcohol, it also dissolves easily in ether, and moderately well in light petroleum. In these respects it differs markedly from the other basic hydrolysis products of the proteins. It was purified by recrystallization from light petroleum. A small amount, possibly a dibenzoyl derivative, did not dissolve. After two recrystallizations a product of constant m.p. 68°-69° C. was obtained in the form of white clusters of small needles. The analyses leave no doubt that the substance is a tribenzoyl derivative.

0.1035 g gave 0.2600 g.  $\text{CO}_2$  and 0.0502  $\text{H}_2\text{O}$ .

0.1143 g. gave 5.52 c.c.  $\text{N}_2$  at 15.5° C. and 763 mm

C = 68.52 per cent, H = 5.39, N = 5.78 per cent.

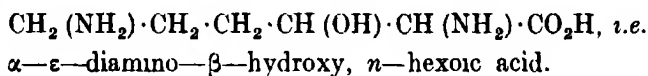
$\text{C}_6\text{H}_{12}\text{O}_3\text{N}_2 (\text{C}_6\text{H}_5\text{CO})_2$  requires C = 64.86, H = 5.94, N = 7.57 per cent.

$\text{C}_6\text{H}_{11}\text{O}_3\text{N}_2 (\text{C}_6\text{H}_5\text{CO})_3$  requires C = 68.35, H = 5.49, N = 5.9 per cent.

*Constitution of the Base.*

The base contains both the nitrogens in the form of amino-nitrogen, as determined by the method of Van Slyke. It also contains a carboxyl group as demonstrated by the fact that its solutions can be titrated by alkalis in the presence of formaldehyde by Sorensen's method. Furthermore, it is highly probable that the third oxygen atom is in the form of a hydroxyl group, as the

base yields a tribenzoyl derivative by the Baumann-Schotten method. It is, therefore, most probably a diamino-monohydroxy hexoic acid. Now several of the hydrolysis products of the proteins exist in pairs, the members of which differ from one another only by a single oxygen atom such as—alanine and serine (hydroxyalanine) ; proline and oxy-proline ; phenylalanine and tyrosine ; glutamic acid and oxyglutamic acid (which latter substance was isolated only a few years ago by Dakin). Analogy, therefore, suggests that oxylysine is a hydroxy derivative of lysine, i.e., it is an  $\alpha$ — $\epsilon$  diamino derivative of  $n$ —hexoic acid. As oxylysine exists in the form of a hydroxy derivative and not of a lactone, the hydroxyl group is not probably in the  $\gamma$  position. These facts would suggest as the most probable formula for oxylysine the following :—



It will probably require relatively large amounts of material and an extensive research to determine definitely the correct formula of this substance.

#### *Occurrence of the Base in Hydrolysis Products of other Proteins.*

Although the base forms only a relatively small proportion of the hydrolysis products when it is present, its amount can be estimated quantitatively with relative ease, owing to the fact that it is the only base which yields a barium carbamate insoluble in cold water. Two methods of estimation have been adopted.

(i) After the separation of the barium salts of the dicarboxylic acids, the remaining amino-acids are precipitated as barium carbamates. These are extracted with cold water. After decomposition of the insoluble fraction with hot water, the base in the fraction thus obtained is precipitated from acid solution with phosphotungstic acid. The nitrogen in this precipitate indicates the amount of nitrogen present as oxylysine

(ii) The total bases from the hydrolysis products are precipitated by phosphotungstic acid. The phosphotungstates are decomposed in the ordinary way, and the bases are then precipitated in the form of the barium carbamates, which are then extracted with ice-cold water. The nitrogen in the insoluble portion is then estimated.

Both methods give the same result. Where the estimation of the oxylysine only is required, the second method is the more rapid. Oxylysine has been isolated in mass by Mr. D. I. Evans from the proteins of oats, obtained by extracting oatmeal with dilute alkali, and precipitation of the proteins from

## 64     *Isolation of a Product of Hydrolysis of the Proteins.*

the alkaline solution by neutralisation with acids. The method employed was precisely the same as that used in the preparation of oxylysine from isinglass. The product thus obtained contained 17·41 per cent. N (Kjeldahl) and the molecular weight, determined by titration in the presence of formaldehyde was found to be 164.

0·1358 g. of the base gave 0·2242 g. CO<sub>2</sub> and 0·1098 g. H<sub>2</sub>O, whence C = 45·04 and H = 8·99 per cent.

All these figures are in fairly satisfactory agreement with the theoretical figures for oxylysine, and there is no doubt that this substance, therefore, is amongst the hydrolysis products of the oat proteins. Only in the case of isinglass and of the alkali-soluble proteins of the oat has the base up to now been isolated in mass. Its amount, has, however, been determined quantitatively in other proteins. The results are given in the following table, which gives the nitrogen of the oxylysine, expressed as the percentage of the total nitrogen of the protein :—

Protein.	Source.	Base N (Percentage of total protein N).
<i>A. Animal origin—</i>		
Casein ..	Cow's milk .. .. .	0
Fibrin . .	Horse blood ....	small trace
Albumin ..	Hen's egg ... . . . .	0
Gelatin	Coignet's gold label .. ..	< 0·3
Gelatin .	Fish skins (cod and ling) .	1·8—2·2
Isinglass	Swim-bladder of sturgeon . .	2·98—3·3
<i>B Vegetable origin—</i>		
Alkali-soluble ..	Oats..... . . . .	1·50
Albumin .. .	Cabbage leaf .. . . .	1·55
Edestin ... ..	Hemp seed... . . . .	3·28

### *Summary.*

By means of the "carbamate" method, a base C<sub>6</sub>H<sub>14</sub>O<sub>3</sub>N<sub>2</sub> has been isolated from isinglass. It differs from the other basic products of hydrolysis of the proteins, in that it yields a barium carbamate insoluble in water. It gives a tribenzoyl derivative C<sub>6</sub>H<sub>11</sub>O<sub>3</sub>N<sub>2</sub> (C<sub>6</sub>H<sub>5</sub>CO)<sub>3</sub> m p. 68°—69° C. Reasons are given for assigning to the base the formula (H<sub>2</sub>·(NH<sub>2</sub>)·CH<sub>2</sub>·CH<sub>2</sub>·CH(OH)CH<sub>2</sub>·(NH<sub>2</sub>)CO<sub>2</sub>H.

It has been found, up to the present, amongst the hydrolysis products of fish gelatin, isinglass and three vegetable proteins of very diverse origin. It is absent from, or present only in very small traces in the hydrolysis products of gelatin of mammalian origin, of casein, fibrin and egg-white.

Part of the above work was carried out under the auspices of the Adhesives Committee of the Department of Scientific and Industrial Research.

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*Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen. Part IX.—Muscular Activity and Carbohydrate Metabolism in the Normal Individual.*

By K. FURUSAWA.

(Communicated by Prof. A. V. Hill, F.R.S.—Received January 12, 1925)

(From the Department of Physiology, University College, London)

(1) INTRODUCTION.

It has long been discussed what substance is primarily responsible for the provision of energy in muscular contraction. The protein hypothesis of Liebig was abandoned in his later years. It was found that the greater excretion of uric acid, or the appearance of creatinin after severe exercise, was merely due to the splitting of the components of muscular tissue, *i.e.*, protein is not used for the provision of energy.

Chauveau (1) assumed the conversion of fat into carbohydrate and a 30 per cent. loss of energy in this conversion. Chauveau's conclusion was discussed by Zuntz (2). He put forward the assumption that the muscles, whether resting or active, utilise fat and carbohydrate in the proportion in which these are presented to them.

A great amount of work has been done by Atwater and Benedict (3), by Zuntz (4), by Benedict and Cathcart (5), and by Campbell, Douglas and Hobson (6), confirming to some degree the view taken by Zuntz. Briefly, they found that both carbohydrate and fat may be used, in a similar manner, as a source of energy in muscular contraction, but that muscles display a marked preference for carbohydrate. The problem has recently been attacked from another standpoint, that of the lactic acid formation and removal in the isolated muscle,

especially by Fletcher and Hopkins (7), A. V. Hill (8), Meyerhof (9), and their co-workers. Both on chemical and on physical grounds, the conclusion reached was that carbohydrate (or, rather, glycogen) is the sole substance, by the breakdown of which the muscles obtain the energy of contraction. Winfield (10), moreover, found no change in the fat-content of the isolated frog's muscle, after complete fatigue and recovery. These experiments were made on the isolated muscle, and were open accordingly to the objection that in the isolated muscle some mechanism may be absent which in the intact animal allows fat to be oxidised.

In 1920, Krogh and Lindhard (11) tried to solve this discrepancy between the two schools by relating the mechanical efficiency to the respiratory quotient during exercise. Employing a method in which the respiratory quotient could be determined with an accuracy of 0.002, by measuring the cost of maintaining a given moderate intensity of work in highly trained subjects, and by varying the substances metabolised by varying the diet, they found the cost of work (*i.e.*, the total amount of energy used in doing a given amount of work) to be a linear function of the respiratory quotient, falling as the respiratory quotient rose. If we assume that the carbohydrate oxidised is utilised directly for work, or, more accurately, for recovery from work, and that fat may be so used only after a conversion involving metabolic processes and loss of energy, then the cost of work should be a linear function of the respiratory quotient. As the result of very careful experiments these workers found, assuming carbohydrate to be utilised directly, that fat may be used only after "conversion" involving a 10 per cent. loss of energy: in modern terms, the recovery process is 10 per cent. less efficient when fat is oxidised than when carbohydrate is oxidised. Their work suggested that the primary breakdown is that of carbohydrate, and that fat is used in a secondary manner to restore carbohydrate which has disappeared.

The results obtained in the last few years by the Cambridge school of physiologists have changed our point of view on this problem. To quote Fletcher and Hopkins (12), "in the evolution of the muscle it would appear that advantage, so to speak, has been taken of this acid phase in carbohydrate degradation, and that by the appropriate arrangement of the cell elements, the lactic acid, before it leaves the tissue in its final combustion, is assigned the particular position in which it can induce those tension changes upon which all the wonders of animal movement depend."

Now consider the two kinds of heat engine. In the external combustion engine, such as the steam engine, the substance which expands by heat and

produces the mechanical work has no relation, at least in a chemical sense, to the substances combusted. In the internal combustion engine, however, the relation is different, *i.e.*, the substances which drive the machinery are the oxidation products of the combusted substances. Thus, in the latter engine, petrol or some other liquid fuel may be used for providing the power, while coal or wood may not be so used. In order to use wood or coal in the internal combustion engine, they must first be distilled, and in this operation a part of their energy is wasted, at least as regards its work-producing power. Putting our problem in terms of the modern theory of muscular contraction, and assuming that the initial process in contraction—that which causes the mechanical response—is absolutely a non-oxidative one, *viz.*, the formation of lactic acid from glycogen, we are asking whether the recovery process by which the lactic acid is restored to its precursor can go on at the expense of any oxidation, or only of that of carbohydrate.

Krogh and Lindhard's results point to the conclusion that carbohydrate is the sole substance which gives the energy to the muscle, while fat is used only indirectly, *e.g.*, to replenish the carbohydrate store.

## (ii) THE RESPIRATORY QUOTIENT DURING EXERCISE.

It has long been known that the respiratory quotient rises during exercise, though with moderate exercise it may remain at the same level as at rest. In long-continued exercise the respiratory quotient is not unity—it varies with the diet. Campbell, Douglas, and Hobson (6) have shown that during exercise the respiratory quotient frequently approaches unity, while in some cases it does not rise appreciably. Benedict and Cathcart (5) also observed the same phenomena. These facts, however, do not answer our question. The combustion of carbohydrate, followed by a reformation of that carbohydrate from fat or protein, would affect the respiratory quotient in a manner exactly similar to the direct combustion of fat or protein.

During exercise of short duration the respiratory quotient is, in general, a function of the vigour of the exercise. In fig. 1 the abscissa shows the speed (*i.e.*, the number of steps per minute), and the ordinate the respiratory quotient. The fore-period of each exercise was three minutes, and the time of collection was one minute. It is clear from this figure that in very moderate exercise it remains at the same level as at rest, and that it rises with the speed up to 1.5, where the ventilation per minute, in a subject of 53 kilos, is about 110 litres. This fact was also shown, in the case of running, by A. V. Hill, Long, and Lupton in a previous part of the present series (Part VII, p. 157).

Campbell, Douglas, and Hobson have shown that after the exercise the respiratory quotient rises temporarily. This has been further emphasised by

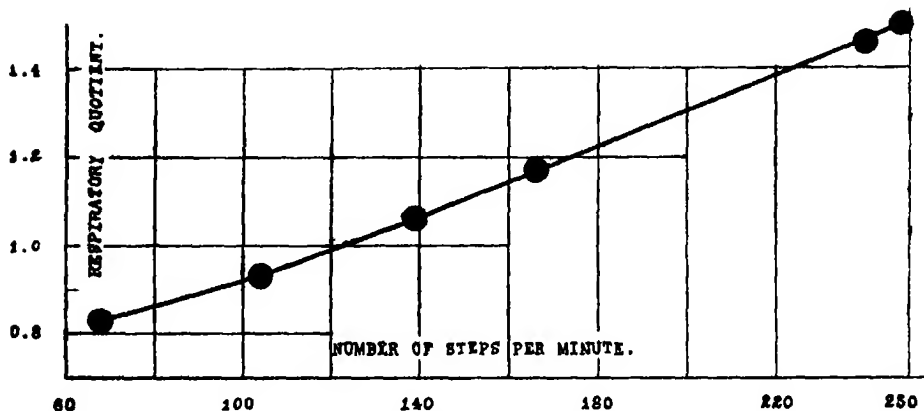


FIG 1.—Respiratory quotient during the fourth minute of exercise, as a function of speed.

Hill, Long, and Lupton (Part III, p. 467). The following observation presents another good example of this phenomenon :—

*Subject* : R. A., “standing-running” 228 steps per minute for three minutes

Time of Collec- tion.	Last Minute of Exercise.	0-10 sec after Exercise	10-30	30-50	50-75	75-105.	105-150.
Respiratory quotient.	1 28	1 32	1.42	1 54	1.62	1 54	1 53

In the later stage of recovery from severe exercise the respiratory quotient may fall as low as 0.6, as shown in Part III of the present series

These large variations in the respiratory quotient—which are all due to the fluctuation of lactic acid in the body—show how necessary it is to exercise the greatest possible precautions if we wish to draw any conclusions, from the respiratory quotient, as to what substances are being oxidised. If these precautions be not taken, the results obtained from the respiratory quotient may show us merely the fluctuations of lactic acid in the body during exercise and in recovery therefrom.

### (iii) METHOD.

The method of Krogh and Linhard can be applied only to exercise of limited vigour, and the conclusions derived from it are indirect. There is, however,

another method of approaching the problem, viz., that described in the previous part on the measurement of the oxygen requirement of exercise (13). In this method the subject may perform exercise of any kind and of any duration, and the gaseous exchanges are followed during not only exercise but recovery. In the present paper "standing-running" was employed, partly because it is easily carried out in the laboratory, partly because it can be performed with any degree of vigour. The subject of the experiments rested for 20 to 30 minutes. During this period the recovery process from the previous muscular movements of ordinary life should be completed. After this, a 10-minute sample of expired air was collected. This gave the *first* basal value. The exercise was then taken. The speed, i.e., the number of steps per minute, was kept constant by a metronome. The whole expired air, from the beginning of exercise until the end of complete recovery, was collected in a large Douglas bag. Another 10-minute collection of the expired air was then taken after the recovery was over; this gave the *second* basal value. These two values give the basal line from which the excess metabolism due to exercise may be calculated: it is necessary to subtract from the total oxygen and the total carbon dioxide the quantities of those gases which would have been used, or expired, in the same time had the subject remained at rest throughout.

To collect the expired air, several Douglas bags of different capacities were used in the various experiments, such as 150 and 500 litres wedge-shaped, and 1,000 and 2,000 litres cube-shaped. The analysis of the expired air was performed at first by the Haldane apparatus as modified by D. T. Harris, and later by mechanically operated apparatus. The subject for the experiments must be fully trained in the method and exercise, or the results will be erratic. It is usually found that when an untrained subject is used his breathing is abnormal when he puts on the mouth-piece and nose-clip. The subjects chosen, therefore, were the author and J. L. P.

#### (iv) RESULTS.

The experiments were divided into two categories, one on subjects on a normal diet, the other on subjects on a fatty diet. Each category contained observations on short and on long-continued exercise.

The short exercise is particularly interesting, since the effort is so small that one might expect it to produce no change in the general metabolism of the body as a whole, but only in that of the muscle. In this case, therefore, we might be able to detect what substance is involved in the primary process of oxidation following muscular contraction. In the case of long-continued



exercise, on the other hand, this primary process may be disguised by the transformations which take place between the different food constituents, in the metabolism of the body as a whole.

(a) *Normal Diet : Short Exercise.*

Table I.—Normal Diet.

Speed : Steps per Min.	Sub- ject	Mean Basal Value CO <sub>2</sub> /O <sub>2</sub> , c c per Min.	Basal R Q	Duration of Exercise, Mins	Time of Collec- tion, Mins	Total Meta- bolism. CO <sub>2</sub> /O <sub>2</sub> , c c	Excess Meta- bolism CO <sub>2</sub> /O <sub>2</sub> , c c.	R Q. of Excess Meta- bolism.
64	F.	231/282	0.82	0.58	10	2795/3310	485/490	0.99
92	F.	181/194	0.93	0.5	10	2165/2290	355/350	1.01
120	F.	210/254	0.83	1.0	20	7658/8446	3458/3366	1.02
146	F.	180/225	0.80	1.0	20	5720/6537	2120/2037	1.04
146	F.	237/280	0.85	1.0	15	6680/7385	3125/3185	0.98
146	F.	229/254	0.90	0.83	20	7720/8253	3140/3173	0.99
160	P.	199/234	0.85	1.0	31	10354/11302	4185/4048	1.03
160	F.	212/245	0.87	0.83	25	7860/8617	2560/2492	1.02
180	F.	222/261	0.85	1.0	33	12203/13545	4877/4932	0.99
182	P.	226/278	0.82	1.0	40	14447/16588	5407/5468	0.99
200	F.	227/275	0.81	0.5	20	7456/8304	2916/2884	1.01
240	P.	240/290	0.85	0.5	30	11538/12751	4158/4051	1.02
244	F.	197/233	0.85	0.5	26	9878/10599	4756/4541	1.05

The duration of exercise varied from 0.5 to 1.0 minute, and the time of collection of the expired air during, and in recovery from, exercise, varied from 10 to 30 minutes, according to the severity of the exercise. The basal values shown in the third column are the averages of those taken before exercise and after recovery. Those two values either coincided exactly with each other, or differed only to a small degree. The last column but one shows the excess metabolism due to exercise, together with complete recovery from it. The most striking result, as the last column shows, is that the respiratory quotient of the excess metabolism is about unity—in fact, the average value is 1.01—and the average difference from unity is quite small (0.02). This is the more remarkable since it holds from the lowest speed, at which the oxygen requirement is only 700 c.c. per minute, to the highest, where an oxygen requirement of 9,000 c.c. per minute is recorded.

The experiments of Table I are in exact accordance with Meyerhof's results on the isolated frog's muscle. He found that the respiratory quotient of the recovery process is about unity, i.e., the complete cycle of the process of exercise in the isolated muscle involves only carbohydrate. The shortness of the period of exertion in Table I practically allows the human muscle to act as an isolated

muscle. From these experiments the hypothesis generally accepted, that muscles, whether resting or active, may utilise fat and carbohydrate in the proportions in which they are presented to them, must be abandoned—at least, for exercise of short duration.

(b) *Normal Diet : Long-continued Exercise.*

The conclusion reached from the above experiments is supported by observations made in the case of very moderate exercise of longer duration (see Table II).

Table II—Normal Diet.

Speed : Steps per Min.	Sub- ject.	Mean Basal Value, CO <sub>2</sub> /O <sub>2</sub> , c c per Min	Basal R Q	Duration of Exercise Mins	Time of Collec- tion Mins	Total Meta- bolism CO <sub>2</sub> /O <sub>2</sub> , c c	Excess Meta- bolism CO <sub>2</sub> /O <sub>2</sub> , c c	R Q of Excess Meta- bolism
120	F	173/202	0.85	10	60	19098/21016	8718/8896	0.98
120	F	208/258	0.80	15	60	22889/25957	10480/10477	1.00
120	F	240/273	0.89	22	55	27627/30144	14427/15129	0.96
120	F.	212/245	0.87	30	70	35065/37495	20225/20345	0.99

The average oxygen intake at the speed employed (120 steps per minute) is of the order of 700 c.c. per minute. Even in 30 minutes of exercise, where 140 gr. of carbohydrate must have been oxidised, the substance used, during exercise and in recovery therefrom, is exclusively carbohydrate. The next series of experiments was done at a higher speed (146 steps per minute)

Table III—Normal Diet

Speed · Steps per Min	Sub- ject.	Mean Basal Value CO <sub>2</sub> /O <sub>2</sub> , c c per Min	Basal R Q	Duration of Exercise Mins.	Time of Collec- tion Mins	Total Meta- bolism CO <sub>2</sub> /O <sub>2</sub> , c c	Excess Meta- bolism CO <sub>2</sub> /O <sub>2</sub> , c c	R Q of Excess Meta- bolism.
146	F	178.206	0.86	15	77	39375/41825	25669/25963	0.99
146	F.	178/216	0.82	20	110	60887/66076	41307/42206	0.98
146	F	180/218	0.82	28	63	60863/66636	49523/52902	0.94
146	F.	165/193	0.85	30	105	66746/76494	49421/56229	0.88

At this speed the oxygen requirement reached, on an average, 1.9 litres per minute. Even in this case the result obtained in the previous experiments may be observed until the duration of exercise extends to 20 minutes. After

this period the respiratory quotient of the excess metabolism falls slowly and indicates that fat is now being called upon for oxidation ; it should be noted, however, that the oxidation of fat can be detected only after 20 minutes of exercise, and *following the oxidation of about 300 gr. of carbohydrate*. This result is in accord with the generally observed fact that in prolonged exercise the respiratory quotient rises at the beginning of exercise and falls slowly as the exercise is prolonged. These experiments were performed under normal conditions, in which a plentiful supply of carbohydrate was taken in the diet. The respiratory quotient at rest was usually in the neighbourhood of 0.85.

(c) *Fatty Diet.*

Fatty diet consisted mainly of fatty meat, butter, and a small amount of watery vegetables.

In order to consume some of the previously accumulated glycogen, exercise was taken. A description of the body condition in the case of the fatty diet is as follows :—

*Subject, K. F.*—After five fatty meals the subject took a walk of 5 miles, and another of 1 m<sup>ile</sup>. On the evening of the third day he felt very weary, but otherwise no bad sign. The experiments were done on the morning of the 4th and 5th days. After lunch on the 5th day he felt bodily disturbances, such as general weariness, aching in the back, and afterwards there suddenly appeared two fatty tumors on the chin and neck. The first series of experiments was then stopped

Table IV.—Fatty Diet.

Speed : Steps per Min.	Sub- ject.	Mean Basal Value. CO <sub>2</sub> /O <sub>2</sub> , c c per Min.	Basal R Q.	Duration of Exercise Mins	Time of Collec- tion. Mins	Total Meta- bolism. CO <sub>2</sub> /O <sub>2</sub> , c c.	Excess Meta- bolism. CO <sub>2</sub> /O <sub>2</sub> , c c.	R.Q of Excess Meta- bolism
90	F	205/284	0.72	1 0	20	5087/6674	987/994	0.99
140	F	185/259	0.71	1.0	20	6101/7443	2401/2263	1.06
146	F	212/282	0.75	2 0	30	9380/11424	3020/2964	1.02
146	P	169/231	0.73	1 0	26	8008/9663	3302/3345	0.99
162	P	188/250	0.75	1 0	24	9769/11256	5255/5256	1.00
178	F.	177/271	0.65	1 0	35	11039/14071	4844/4586	1.06
184	P.	224/288	0.77	0 5	25	8420/10011	2820/2811	1.00
196	P	204/266	0.77	0 5	25	7232/8616	2132/1966	1.08
216	P.	204/266	0.77	0 41	22	7745/9193	3257/3341	0.98
248	F	209/274	0.76	0.5	35	10004/11224	2689/2634	1.00
264	P	238/317	0.75	0.25	20	7300/8760	2540/2420	1.04
272	F	204/285	0.71	0 33	22	7221/8839	2733/2569	1.06

In the sixth line of Table IV a respiratory quotient of 0·65 in the basal value is recorded. A description of that experiment follows :—

*Subject, K F—December 8, 1924.*

Breakfast.—One cup of tea, without sugar and milk.

Lunch.—Fat part of ham, cheese roasted with butter.

Supper.—Fat soup, then the same diet as lunch

In the afternoon walked 6 miles and 1 mile.

Heavy feeling on the shoulder Indigestion.

December 9.—1 mile walk in the early morning ; heavy weary feeling. No appetite.

No meals. This afternoon a respiratory quotient of 0·65 was reached.

*Protocol of the experiment—*

Speed · 178 steps per minute for 1 minute.

Basal value before exercise—

Oxygen	... ..	269 c.c. per minute.
Carbon dioxide.....	. . . . .	175 c.c
Respiratory quotient	.. . . .	0·65.
Ventilation per minute	. . . . .	4·36 litres

Total bag (exercise plus recovery)

Duration of exercise	.....	1 minute.
Time of collection	....	35 minutes.
Ventilation	. . . . .	266 litres.
Oxygen used	.. . . .	14,071 c.c.
Carbon dioxide expired	. . . . .	11,039 c.c

Basal value after recovery—

Oxygen	. . . . .	274 c.c. per minute.
Carbon dioxide.	.....	179 c.c
Respiratory quotient	.. . . .	0·65

Excess metabolism (taking the mean basal value)—

Oxygen used	. . . . .	4,586 c.c.
Carbon dioxide	. . . . .	4,844 c.c
Respiratory quotient	. . . . .	1·06.

From the table we can see that on the fatty diet, even when the respiratory quotient of the basal value reaches 0·71 (which is regarded as an indication of the combustion of fat only), the excess metabolism produced by short-lived exercise and complete recovery therefrom is almost exactly unity, as already shown on the normal diet. That is, the metabolism due to a short element of exercise is the same, viz., the oxidation of carbohydrate only, whether a great amount of carbohydrate, or only fat, is supplied to the body. This suggests that the position of carbohydrate in muscular contraction is unique, and that fat is used only after conversion into carbohydrate. Lusk (14) studied the D : N ratio in the phlorhizinised dog, and found that, while sugar is derived from protein, it is not derived from the metabolism of fat. If the above

conclusion be correct, we can only assume, in this case, that the animal under phlorhizin treatment has lost its power of forming carbohydrate from fat.

In a recent paper, Meyerhof and Himwich (15) have shown that in mammalian (rat) muscle on a fatty diet, the glycogen content falls considerably as compared with that in the normal animal, and that in such muscles the tension developed in rigor is smaller than in the normal. They conclude that lactic acid only is responsible for muscular activity, and that this cannot be replaced by any other acid. Moreover, Himwich, Loebel and Barr (16) have found that the lactic acid formation in the diabetic individual is just as much the basis of muscular contraction as in the normal one

(d) *Fatty Diet : Long-continued Exercise.*

Table V—Fatty Diet

Speed : Steps per min	Sub- ject	Mean Basal Value CO <sub>2</sub> /O <sub>2</sub> , c c. per Min	Basal R Q	Duration of Exercise Mins	Time of Collec- tion Mins	Total Meta- bolism CO <sub>2</sub> /O <sub>2</sub> , c c	Excess Meta- bolism CO <sub>2</sub> /O <sub>2</sub> , c c	R Q of Excess Meta- bolism.
146	P	188/250	0.76	5	35	19486/22525	12906 13775	0.94
146	F	215/276	0.77	4	43	19770/22690	10525 10822	0.97
146	F.	139/192	0.72	7	50	20876/24745	13926 15145	0.92
146	F.	158/208	0.76	9	100	34713/40737	18913 19937	0.95
146	F	180/235	0.76	9	88	34675/41100	18835 20420	0.92

In long-continued exercise, a speed of 146 steps per minute was adopted. It is clearly seen from Table V that fat is more quickly called upon than on a normal diet. This is shown in fig. 2, in which the dotted line represents a

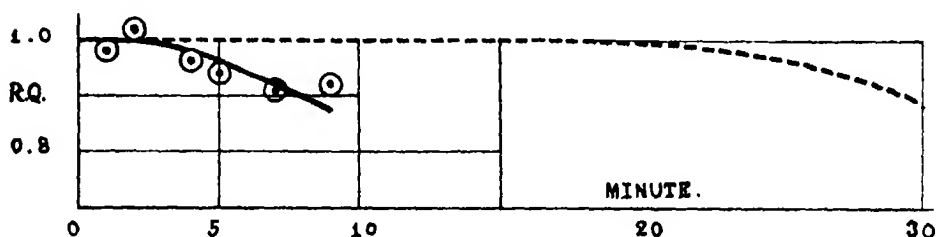


FIG. 2.—Respiratory quotient of the excess metabolism due to exercise of various durations; broken curve normal diet; full curve through the observations, fatty diet.

normal diet, while the smooth line through the observed points represents a diet of fat. Even in 9 minutes exercise, during which the subject felt as

though he had walked 20 miles, the proportion of carbohydrate decomposed was considerably greater than that of fat.

#### SUMMARY.

The problem of the source of energy in muscular contraction was studied on normal human subjects, by determining the respiratory quotient of the excess metabolism during not only exercise but recovery. The substance being oxidised at rest was changed by varying the diet. A discussion of the respiratory quotient during exercise (not including recovery) is given, and it is shown that it is impossible to determine what substance is being oxidised, in the muscle itself during exercise, from the respiratory quotient. The results obtained are .—

(1) On a normal diet, the average value of the respiratory quotient of the excess metabolism due to a short element of exercise is 1.01 ; i.e., carbohydrate only is responsible for the process of contraction and recovery from it. As the duration of exercise is prolonged the respiratory quotient of the excess metabolism falls slowly, and indicates that some substance other than carbohydrate is being called upon

(2) On a fatty diet, it is shown that even when the basal respiratory quotient reaches 0.71, short-lived muscular exercise is performed at the expense only of carbohydrate, as on the normal diet. In long-continued exercise, however, fat takes part more quickly than on the normal diet. From these facts we can conclude that in exercise of short duration, in which no change in the general metabolism of the body as a whole might be expected, the human body acts as though it were an isolated muscle, in which carbohydrate is the only substance oxidised, as shown by Meyerhof. The primary fuel of contraction, therefore, in the human muscle is carbohydrate, and fat or protein is presumably used to replenish the carbohydrate store which has disappeared.

It is a great pleasure to acknowledge my indebtedness to Prof. A. V. Hill and Mr. C. N. H. Long, and to Mr. J. L. Parkinson, who willingly underwent the severe exercise and the unpleasant experiences of a fatty diet.

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*The Origin and Destiny of Cholesterol in the Animal Organism.*  
*Part XIV—The Cholesterol Metabolism in Normal Breast-Fed Infants.*

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(Communicated by Sir Walter Fletcher, F.R.S — Received 16 January, 1925.)

(A Report to the Medical Research Council from the Biochemical Department,  
 St. George's Hospital.)

In Part XII (1921) of this series of papers, we gave an account of our investigations on the cholesterol balance of normal adult men fed on a known and carefully controlled diet. In the 26 cases examined the average intake of cholesterol per day was 0.253 grm. and the average output 0.56 grm. The average negative balance was thus 0.307 grm. per day, the individual values varying between +0.05 (1 case) and -0.9.

From these experiments we were led to the conclusion that since cholesterol is an integral constituent of all cells of the body and there is an excess of output over intake, *there must be some organ in the body capable of synthesising cholesterol.*

The balance in the above experiments was in reality the balance of the sterols precipitable by digitonin.

The sterols ingested with the food consisted largely of cholesterol and its esters, but as some of the diets contained brown bread, small quantities of sitosterol and perhaps other phytosterols would be present, with probably also traces of bihydrostitosterol (Anderson, 1924). The sterols of the faeces consisted mainly of coprosterol with smaller quantities of  $\beta$ -cholestanol and cholesterol. Traces of sterols of vegetable origin may also have been present.

The adult human subject is marked off from other animals by the fact that the cholesterol passed into the intestine in the bile undergoes reduction at some stage to coprosterol and  $\beta$ -cholestanol, probably by bacterial action, though a small quantity of cholesterol escapes this process (Gardner, 1921). It would seem a probable assumption that this reduction limits the re-absorption of the cholesterol, according to the hypothesis of the cholesterol-cycle propounded in earlier papers of this series. This has not yet been definitely proved, though unpublished experiments by one of us on rabbits indicate that coprosterol fed to these animals is very largely, if not entirely, excreted unchanged.

It was shown by Müller (1900) that prolonged feeding on a milk diet clears the intestine of organisms which effect this change, and that then cholesterol is excreted as such, and unaccompanied by coprosterol.

It would have been impracticable to treat healthy adults in this way, for obvious reasons. It is well known, however, that infants in the milk stage excrete only cholesterol, and it seemed worth while repeating the experiments, using breast-fed, or milk-fed infants, in hope of throwing more light on the subject. The use of infants appeared to have the following important advantages :— (1) a single food, capable of easy and accurate analysis, (2) stools in which the only sterol is cholesterol (or its esters); (3) no interference with the ordinary diet of the child; (4) little or no interference with the usual mode of life of the mother or child; (5) the experiments are made at a stage at which active growth is taking place.

*Previous work on this subject.*

In recent years several papers have appeared on this subject. In 1919 Beumer described two experiments in which intake of cholesterol was balanced against output. Both infants were breast-fed and were presumably healthy but they were decidedly below the average in weight. The following year a similar series of estimations was published in America by Gamble and Blackfan (1920), for the express purpose of testing whether cholesterol could be synthesised in the infant organism. Four subjects were used, and each experiment lasted three days, the experiment faeces being marked by the administration



of a little charcoal at the beginning and end. Unfortunately the infants were admitted to be somewhat abnormal in health, as they were "convalescing from more or less serious nutritional disturbances," and were much below normal weight for age. During the investigation, however, they were stated to be in good health, lively and gaining weight. Only one child received breast milk and this was given by bottle; the others had a modified milk, made up of cow's milk, barley water and cane-sugar. In every case a negative balance was observed, so that the authors concluded that synthesis was taking place.

A more elaborate series of experiments was published by Wacker and Beck in 1921. They carefully estimated the intake and output, both of fat and cholesterol, in several children. Apparently only two of the infants were entirely breast-fed, and the other two, on which most of their experiments were made, are described as "markedly atrophic and hypertonic." They each received modified diets and the results were rather complicated by the occasional addition of free cholesterol to the diet.

Both Beumer, and Wacker and Beck assume that synthesis by the organism is either impossible or of quite secondary importance. They even go so far as to estimate the amount of cholesterol that must be added to the diet in order to avoid a negative balance. Output, they maintain, is adjusted to intake by means of a "regulating mechanism," which according to Wacker and Beck is closely bound up with fat metabolism, but according to Beumer is comparable to the "adjustment of intake and output of nitrogen."

A further paper, on similar lines, was published by Beumer in 1922. Various diets were used, and, in general, the results were held to confirm his hypothesis of a regulating factor that adjusts output according to intake.

For lack of experimental details all these results are not easy to compare with our own, but they appear to show that larger amounts of sterol added to a diet are only partially absorbed, and quickly reappear in the stools, and that when the intake is very low the output is also at a minimum.

From this brief account, it would seem that, though a good deal of valuable work has been done on sterol metabolism in infants, we are still without reliable data dealing with the intake and putput of cholesterol in perfectly healthy children under quite normal conditions. That such data must form the basis of any study of pathological cases gives an additional interest to the experiments to be described.

*Selection of cases and procedure.*

We worked only with children medically certified to be quite healthy and normal. Usually they were confinement cases, at the General Lying-in Hospital, York Road; the children's ward, St. George's Hospital; Maternity Department, Kensington Infirmary; the Royal Free Hospital; and the Mothercraft Training Centre, Earl's Court.

Care was taken to interfere as little as possible with the normal life of either mother or child. The only interference necessary was the regular weighing of the child before and after each feed, and the drawing-off of samples of milk from the mother. Most of the mothers went out of hospital at the end of the second week, or thereabouts, and only in a few cases was it possible to deal with later periods.

*Estimation of cholesterol intake*

For an exact determination of the amount of cholesterol consumed by the child, it is obviously only necessary to measure accurately the quantity of milk taken and its cholesterol contents. Our earlier experiments, however, soon showed that this was a more difficult problem than would appear at first sight.

A careful survey of the literature on the composition of human milk showed that the percentages of fat, protein, carbohydrate and mineral matter are much more variable in different normal subjects than the text-books would lead us to suppose, and this variability was particularly marked in the case of fat. In addition to this, the composition of milk of the same individual shows progressive changes with duration of lactation, and, when consecutive samples are withdrawn, even from the same breast. Even the time of day at which samples are taken appears to have some influence, and whether milk is drawn from the right or left breast.

It was therefore necessary, before undertaking much of the work with which this paper deals, to make a detailed study of the cholesterol content of both cow and human milk, from as many different sources as was possible and under a variety of conditions, and ascertain the limits of variation in different normal individuals (human), the changes with duration of lactation, the effect of progressive milking, etc.

The results of this investigation have been published in detail in the *Biochemical Journal*, cow's milk in 1923 and human milk 1924. For the sake of space we do not summarise the results here, but we may state that they form a complete experimental justification of the methods of sampling adopted in this paper. Either of the following methods proved satisfactory and inter-

ferred but little with the comfort of the mother. Adequate, approximately equal, samples were withdrawn just before and after nursing, and the analysis was made on the mixed samples. In some cases we analysed a sample composed of several portions of mid-nursing milk taken throughout the 24 hours.

In order to determine the weight of milk consumed by the infant, it was weighed before and after nursing on balance sensitive to under quarter of an ounce. For all very young infants this was done at each nursing during the 24 hours, the total amount being then multiplied by the cholesterol value of the sample. In one or two cases of older children it was found that the intake of milk was very regular, and consequently we considered that it was sufficiently accurate to make a "test weighing," *i.e.*, a series of weighings at each nursing during the 24 hours prior to and during the 24 hours following the period of experiment, and then assume the intermediate days had been on a par. Details in these cases are given.

The cholesterol was estimated by the method described by Fraser and Gardner (1910) with some slight modifications detailed in our papers on cow's milk (1923) and in a critical study of the methods of estimating cholesterol (1924 (2)).

#### *Cholesterol output.*

In experiments of this kind it is often the practice to mark the beginning and the end of the experimental period by administering some non-assimilable material such as charcoal, but in dealing with infants such a procedure was felt to be undesirable. In our previous experiments with adults no special demarcation of the excreta was attempted, but to eliminate any effect of previous alimentation, the data obtained from the first three days of each experimental period of 10 days were excluded from the final calculations. In these infant experiments such special demarcation was even less necessary, since the evacuations were quite regular and the ordinary diet was not being interfered with in any way.

In the earlier experiments the fæces were collected on small ordinary napkins, but a later improvement, in order to reduce the bulk to more manageable proportions for extraction, was to use thin squares of cotton lint, which could be constantly renewed. The napkins with fæcal matter were thoroughly dried at 100 degrees and then cut into small pieces. It was generally possible to reject a large amount of unsoiled lint, but care was taken to ensure that none of the fæces were lost. The whole was then extracted with ether for 7 to 10 days in large metal Soxhlet's apparatus. The ether extract as a rule was bright golden yellow, and contained all the fat, free fatty acid and sterol. The total

ether extract was estimated and the whole was then saponified by addition of a large excess of an alcoholic solution of sodium ethoxide, in the manner described in our former papers. The unsaponifiable matter was obtained free from soap and the cholesterol estimated as already described.

*Cholesterol excretion during the first week of life*

Though we made estimations of the cholesterol output during the early days of life, we made no direct determinations of the cholesterol balance during this period, partly because it was not always considered desirable to interfere with the mother at this stage, but mainly because the normal fæces are more or less mixed with the pre-natal contents of the intestines, usually referred to as meconium. This consists of a dark green tenacious material, about the composition of which very little seems to be known. In an account of its properties Müller (1884) notes that the ether extract contains cholesterol, but we have been unable to find any other analyses giving reliable figures as to the amount. We therefore made some estimations of the amount of cholesterol in the intestinal contents at birth.

(1) 7 months old foetus. Contents of large and small intestines weighed 8.5 gm., and contained 0.0071 gm. cholesterol

(2) Still-born full term foetus (one of twins). 8.2 gm. of a dark green viscous material was obtained from the intestines, which contained only a trace of cholesterol.

(3) Full term still-born foetus. Small intestine (stomach to cæcum) contained 21.9 gm. of a pink gelatinous material, which gave a faint bile reaction. It yielded 0.068 gm. of ether extract, and 0.0055 gm. cholesterol.

The large intestine (cæcum to colon) yielded 62 gm. of matter varying in colour from bright yellow to dark green. It yielded 0.923 gm. of ether extract, 0.855 gm. of unsaponifiable matter and 0.557 gm. of cholesterol.

(4) Full term still-born foetus. Small intestine 18.9 gm. material, similar to No. 3, but deeper yellow in colour. It yielded 0.25 gm. of ether extract and 0.073 gm. cholesterol

Large intestine. The contents were dark green and weighed 60.6 gm. This yielded 1.54 gm. of ether extract, 0.977 gm. of unsaponifiable matter and 0.504 gm. of cholesterol.

In Group I, Table II, we give details of seven infants during the first week of life. The cholesterol in the fæces was estimated as described, but no direct estimations of intake were made. It is, however, possible, from data we have obtained, to calculate with moderate approximation what the probable maximum

Table I.—Comparison of observed and estimated intake of milk and sterol

Age.	In days				In months.									
	1-7	8-14	15-21	22-30	1-2	2-3.	3-4.	4-5	5-6	6-7.	7-8	8-9.	9-10	
Average daily amount of milk taken by normal child in cubic centimetres after Pritchard (1922)	284	330	450	510	600	777	850	900	1,000	1,050	1,100	1,150	1,200	
Average amount of milk taken, in our own experiments	270	417	442	—	—	633	—	—	—	—	—	916	802	
(Number of cases for which averages based)	(4)	(5)	(1)	—	—	(3)	—	—	—	—	—	(2)	(1)	
Average amount of milk observed by Wacker, Beumer and Gamble	—	—	342	—	610	452	667	720	396	—	—	—	—	
(Number of Cases)	—	—	(1)	—	(1)	(6)	(4)	(2)	(1)	—	—	—	—	
Probable daily cholesterol intake, } Max based on the average cholesterol } content of milk at different } periods of lactation. (Fox and } Gardner) }	0.10	0.13	0.14	0.15	0.16	0.17	0.18	0.19	0.20	0.21	0.22	0.23	0.24	
Observed sterol intake, average actually determined in our experiments	0.05	0.08	0.08	0.09	0.10	0.12	0.12	0.13	0.13	0.14	0.14	0.15	0.16	
(Number of Cases)	0.06	0.15	0.06	—	—	0.13	—	—	—	—	—	0.24	0.21	
Observed sterol intake by Wacker, Beumer and Gamble	(2)	(4)	(1)	—	0.07	(3)	0.07	0.11	0.04	—	—	(2)	(1)	
(Number of Cases)	—	—	0.05	—	(2)	(4)	(3)	(1)	(1)	—	—	—	—	

cholesterol intake over this early period is likely to be. This has been done by means of the data in Table I, which is based on the average amount of milk taken by the "normal" infant at various ages, and on our analyses of milk taken at various stages of lactation. These averages are, of course, only very approximate, since individual variation is large, and age is in fact a much less important factor than the weight and vigour of the child. However, a comparison of the average with the observed values will show that within certain fairly wide limits, it is possible to estimate in this manner, and to obtain a useful idea of, the probable intake.

In Table II the maximum possible intake has been allowed for, assuming that 300 c.c. of colostrum were consumed. This would work out at about 0.1 gm. per day, but it is almost certainly an over-estimate. The average balance it will be seen is decidedly on the negative side, — 0.14. This, however, can be, to a considerable extent, accounted for by the cholesterol of the meconium, but to what extent it is impossible to determine, owing to the very variable quantities of sterol found in the still-born children examined.

Table II, Group I.—Cholesterol balance during the first week of life.

Number of Case	A	B	C.	D	E	F	G
Duration of experiment in days	1	1	3	1.5	3	3	3
Age of child (days)	1	2	2 4	3 4½	3 4	3 5	7-9
Estimated maximum cholesterol intake in gm.	0 10	0 10	0 10	0 10	0 10	0 10	0 10
Output of sterol in faeces in gm	0 095	0 214	0 248	0 283	0 414	0 143	0 206
Balance	0	- 0 11	- 0 15	- 0 18	- 0 30	- 0 04	- 0 2

Average intake, 0 10, Output, 0 24, Balance, - 0 14 gm per day

*Cholesterol balance during the second and third week of life.*

The results of seven cases in which both intake and output of cholesterol were experimentally determined are given in Group II, Table III. The experiments in the group were usually carried out for three days, and cover the period when, according to the classification of Holt (1915), colostrual milk is changing into true milk.

The cases of H and I are on the border line between Group I and Group II, and perhaps ought to come in Group I. They are, however, included in this group as the cholesterol intake was actually determined. On the whole, there

appears to be a decrease in the cholesterol excreted with increasing age, though the intake is slowly rising.

Table III, Group II.—Cholesterol balance during the second and third week of life.

Number of Case	H	I	J	K	L	M.	N.
Duration of experiment in days	3	1	3	3	3	3	3
Age of infant in days	5-7	7	7-9	8-10	8-10	8-10	17-19
Normal weight of child for age in kilog.	3.2	3.2	3.2	3.3	3.3	3.3	3.5
Actual weight in kilog. at beginning	3.2	3.7	4.2	4.1	3.2	3.2	4.9
Change in weight during the experiment in grm.	+50	50	-28	+100	+70	800	126
Weight of milk consumed in grm.	347	331	387	435	464	421	442
Percentage of cholesterol in milk	0.0188	0.0191	0.0323	0.0334	0.0246	0.0441	0.0128
Daily intake of cholesterol in grm.	0.065	0.063	0.125	0.145	0.114	0.200	0.057
Daily output in grm.	0.073	0.265	0.106	0.169	0.081	0.033	0.060
Balance	-0.008	-0.202	+0.019	-0.024	+0.033	+0.167	-0.003

Average intake, 0.110, Average output, 0.112, Balance, -0.002

Out of the seven cases five show a virtual balance, any difference being probably within the errors of experiment. One case I is markedly negative, but this case is on the border line between the two groups, and the value of the experiment is somewhat qualified by the fact that it only lasted one day. The other case shows a marked positive balance. The intake is high and the output very low. In this connection the very considerable gain in weight during the experiment is significant. The average results show an almost exact balance between intake and output.

#### *Cholesterol balance in children aged 7 weeks to 10 months*

In this Group III, Table IV, consisting of older children, the milk was the "normal" milk of Holt's classification, and the results are comparable with those of the other investigators referred to above. The results cover a period of steady development, and it will be seen that the daily amount of milk consumed has more than doubled.

Experiment O—the first actually made—was a remarkably healthy infant living at home, and in this case the faeces only were analysed. The intake was estimated as in Group I, and in this case we believe is a close approximation to the actual intake. There is a small positive balance.

**Experiment R.** This was the only experiment not made on a perfectly healthy child. The case came into hospital for digestive disturbance and consequent loss of weight a fortnight previously, but a satisfactory diet had been found and weight was being regained while the experiment was in progress. The diet consisted of cow's milk with added sodium citrate, together with a little orange juice and water. The milk used was analysed and the intake calculated accordingly. There was a daily positive balance of 0·012 gm.

**Table IV, Group III.**—Sterol balance in children between 7 weeks and 10 months.

Experiment Number	O	P.	Q	R.	S <sub>1</sub>	S <sub>2</sub>	T.
	twins				10 days		
Duration of experiment in days	7	7	7	8	10 days		10 days
Age of child	7 weeks	2 5 months	2 5 months	3 months	8 months	8 months	10 months
Normal weight for age	—	4 6	4 6	5·5	7 8	7 8	8·5
Actual weight in kilog	--	2 8	3·3	4 8	9 1	8 1	9·0
Change in weight during experiment in gm.	—	+98	-49	—	+14	+77	+98
Weight of milk taken daily in gm.	--	550	554	795	927	905	802
Percentage of cholesterol free and combined in milk	—	0 0253	0 0253	0 014	0 0258	0 0270	0 0252
Daily intake of cholesterol in gm	0·13*	0·139	0 140	0 111	0 483 (together)		0 209
Daily output of cholesterol	0 096	0 070	0 059	0 123	0 216 (together)		0 164
Balance	+0 03	+0 069	+0 081	-0 012	0 267 or 0 134 per child		+0 045

Average intake per day, 0·173; Average output per day, 0 104; Balance, +0 069.

\* Estimated

We were fortunate in being able to carry out the experiments P, Q, S and T at the Mothercraft Training Society, Earl's Court. The children were normal and healthy in every way, in fact, they were living at the Home for educational and comparative purposes.

The first two experiments were made on twin brothers, aged 2½ months, and lasted 7 days; during this period the weight of the first rose from 5 lbs. 13½ ozs. to 6 lbs. 1 oz., while that of the other fell from 6 lbs. 12½ ozs. to 6 lbs. 10½ ozs. A sample of the milk taken at the beginning and end of the experiment gave

		Fat.		Total Cholesterol.
Beginning	..	4·87	..	0·0241
End	..	3·66	..	0·0265



The daily intake of fat was 23·4 and 23·6 grm. and the output as fat and free fatty acids 5·5 and 4·7, equivalent to a percentage utilisation of fat of 90 and 91 per cent. respectively. The faeces, however, contained soaps, which expressed as fatty acids were respectively 3·16 and 2·55 grm. per day. This would reduce the percentage utilisation to 77 and 80 per cent. There was in both children an excess of intake over output, showing that some of the cholesterol had been retained. The original intention in experiment S was to carry out a separate 10-day balance on two 8-months old children. Unfortunately, the two ether extracts of the faeces were accidentally mixed, so that the figures show the net result of the two excretions

In this experiment the milk taken by the children was not measured at each feed during the 24 hours, but was based on such a measurement made during the 24 hours before and after the experimental period.

In order to justify this procedure we give the actual figures obtained at the "Test Feed".—

		Nursing.					Total
		6 a m	10 a m.	2 p m	6 p m.	10 p m	
CHILD I							
24 hours before experiment	Weight of milk in ounces	8	6½	6	6½	5½	32½
24 hours after experiment	Weight of milk in ounces	9½	5	5½	5½	6½	32

Previous weekly test feeds made on the same child had given a 24 hour total of 35, 38 and 30 ozs.

We therefore feel safe in adopting the average 32·6 ozs. as a fair estimate of the daily intake over the period.

		Nursing					Total.
		6 a m.	10 a m.	2 p m.	6 p m.	10 p m	
CHILD II							
24 hours before experiment	Weight of milk in ounces	9	7	4½	6	5½	31½
24 hours after experiment	Weight of milk in ounces	7	7½	6½	5½	5½	32

Previous test feeds  $35\frac{1}{2}$ ,  $36\frac{1}{4}$ . The average used for calculating daily intake during the experiment was 31·90. The intake of cholesterol here was found to be quite definitely greater than the output, and this is all the more striking as the experiment lasted a longer time. The last experiment is an attempt to confirm this positive balance by a second experiment on one of the same children No. 1, the second child having unfortunately left the home.

The child was now 10 months old, and had been receiving some cow's milk in addition to the breast milk. It weighed 18 lbs 11½ ozs. at the beginning, and 19 lbs. 2½ ozs. at the end, of the experimental period. Test feeds made before and after the period gave  $29\frac{3}{4}$  ozs. and  $26\frac{3}{8}$  ozs. as the average daily intake. The mean of these was used in our calculations. Altogether during the 10 days, 426 c.c. of additional cow's milk were taken, containing 0·015 per cent. of total cholesterol. The human milk had the following composition:—

	Fat	Total cholesterol.
Human milk at beginning of experimental period . . . . .	6·90	0·0256
Human milk at end of experimental period . . . . .	7·36	0·0246
Mean . . . . .	7·13	0·0252

And the daily intake was—

	Fat	Total cholesterol.
Breast milk, 802 c.c. . . . .	57·2	0·202
Cow's milk, 43 c.c. . . . .	1·6	0·006
	58·8	0·2086

The daily output of fat, *i.e.*, true fat, fatty acids and fatty acids as soap, was 0·82 gm., so that percentage utilisation of fat was 99 per cent. The cholesterol balance was again positive, but not as markedly as before.

In Table V we summarise the results of Wacker and Beck, Beumer, and Gamble and Blackfan, arranged in order of age of infant.

In Wacker and Beck and Beumer's experiments the digitonin method was used for determining the cholesterol, and the results though indicating a slight negative balance are somewhat similar to our own. Gamble and Blackfan used a modification of Autenrieth and Funk's colorimetric method of estimating the sterol. The colour methods are apt to give higher values than the digitonin method, and as Gardner and Williams (1921) have shown are very unsuitable for *fæces*. Their results show a much more marked negative balance.

Table V.

Observer.	Wacker and Beck	Wacker and Beck.	Gamble and Blackfan	Gamble and Blackfan	Beumer	Gamble and Blackfan.	Gamble and Blackfan	Beumer.	Gamble.
Remarks	Breast fed	Fed with breast milk	Diet	Diet	Breast fed	Fed with breast milk	Diet	Breast fed	Cow's milk
Duration of experiment in days	4	3	3	3	3	3	3	9	3
Age of child	15-18 days	47-49 days	3 months	4 months	4 months	4 months	5 months	5 months	19 months
Normal weight for age in kilog	3.5	3.8	5.2	5.9	5.9	6.3	6.5	6.5	—
Actual weight in kilog.	3.0	3.2	4.1	5.1	5.0	4.9	4.9	2.3	6.2
Daily change in weight experiment in grm	+15	-27	+70	+25	+20	+20	+0	+10	+10
Weight of milk taken daily	342	610	360	340	717	675	540	396	1,200
Percentage utilisation of fat	86	88	—	—	—	—	—	84	—
Daily intake of cholesterol	0.05	0.095	0.05	0.072	0.079	0.107	0.07	0.042	0.125
Daily output	0.066	0.102	0.114	0.212	0.093	0.177	0.125	0.075	0.424
Balance	-0.016	-0.007	-0.064	-0.14	-0.014	-0.07	-0.055	-0.033	-0.299

Table VI.—Wacker's experiments on two sick infants.

Child	I.		II.			
	A.	B.	A	B.	C	D.
Remarks	Diet only	Diet A 0.1 gm cholesterol added daily	Diet only	Diet, 0.1 gm. cholesterol added daily	0.1 gm. cholesterol added to diet daily	Diet without cholesterol
Duration of experiment in days	1	3	1	3	3	3
Age of child	2.5 months <sup>a</sup>	2.5 months	2 months 1 week	2.5 months	3 months 1 week	4 months
Normal weight for age in kilog	4.8	4.8	4.6	4.8	5.5	5.9
Actual weight	2.4	2.4	2.3	—	—	2.7
Daily change during experiment in gm	+40	— 7	+ 0	+10	+23	+ 7
Weight of milk taken daily	490	497	420	497	645	764
Percentage utilisation of fat	97	95	77	91	87	92
Cholesterol intake per day	0.068	0.142	0.157	0.154	0.158	0.065
Cholesterol output per day	0.074	0.112	0.199	0.103	0.122	0.083
Balance	-0.006	+ 0.03	-0.042	+0.051	+0.036	-0.018

In the experiments of the above observers few details are given as to what precautions were adopted in determining the cholesterol intake, and in view of our own experience and in light of the data, on average quantities of milk taken by infants, in Table II, it seems permissible to question whether intake was not somewhat under-estimated. It seems not unlikely that some of the cases were in reality showing a small cholesterol absorption.

In Table VI we give the results Wacker obtained by adding cholesterol to the diet in his experiments with the two sick infants.

In experiment I, column A shows the balance without added cholesterol and B the result of adding 0.1 grm. of cholesterol per day. Experiment II on another child shows A the balance without cholesterol, B and C three-day experiments in which 0.1 grm. cholesterol per day was added to the diet, and D, three days without added cholesterol.

These experiments show that the added cholesterol is partially absorbed, or is at any rate not all immediately excreted.

*Qualitative examination of the unsaponifiable matter.*

The whole unsaponifiable matter left after completing our quantitative analyses was collected together. It was dissolved in ether and again treated with alcoholic sodium ethoxide. It was then diluted with water and taken up in ether. The ethereal solution, after thoroughly washing with water, was evaporated. The residue was crystallised from alcohol or acetone, and as much crystalline sterol as possible separated. The remainder was then precipitated by digitonin, and the cholesterol digitonide dissociated in the usual manner in boiling xylene.

All the crystalline sterol was then submitted to careful fractional crystallisation. With the exception of a very small quantity of a white waxy substance, readily soluble in alcohol, the material consisted *only* of cholesterol, which was characterised by its appearance at melting point and its esters. The waxy substance melted at 75 to 76 degrees, and gave no sterol colour reaction. The residual oil, after separating the sterol by digitonin, was taken up in ether, the solution dried and the ether evaporated. The oil obtained, which was of a bright golden yellow colour, was distilled in a vacuum of 1 to 2 mm. It passed over between 120 and 170 degrees, and was evidently a mixture. We had not enough material for a fractional distillation. On treatment in chloroform solution with acetic anhydride and a few drops of sulphuric acid it gave a reddish brown colour, which on standing assumed a dusky brownish green tinge. Combustion showed that it contained 82.95 per cent. carbon and 12.23 per cent. hydrogen.

*Conclusions.*

(1) In the early days of life, when considerable adjustments of metabolism are taking place, colostrum is giving place to true milk, and the meconium is being got rid of, there is a more or less marked excess of output of sterol over intake. During the next two weeks, in which, according to the classification of Holt, milk is still in a transitional stage, there is practically a balance between intake and output, or if anything the balance is slightly on the negative side. As the age advances the balance becomes more decidedly positive, and there is evidence that actual utilisation of the sterol of the diet is taking place.

During the first three months a normal infant will gain as much as 70 per cent. in weight, and it seems obvious that a considerable increase in the total sterol of the body must take place. If the diet were the only source of cholesterol there could, from our results, be no such marked gain, and we think that our experiments fully confirm the conclusion arrived at from the study of adults, namely that *there must be some organ in the body capable of synthesising cholesterol.*

(2) It is now a well-established fact that sterol present in the food can, in the case of both herbivora, such as rabbits, and carnivora, such as dog and cat, be absorbed into the lymph and into the blood stream, and there is no reason to suppose that the case is different in the human infant. The results in Table IV afford evidence of such utilisation. The experiments of Wacker on his two sick children are a confirmation of this, since they show some utilisation of the added cholesterol. We therefore consider the conclusion justifiable, that although synthesis is probable, *the sterol present in the diet is a source of supply which cannot be disregarded*; a conclusion which again emphasises the strict economy exercised by the animal organism in dealing with these substances, an economy amply proved in earlier papers in this series.

In recent years the chemical investigations of Wieland and his co-workers on the bile acids (1912), and of Windaus (1920) on the oxidation of the hydrocarbons, cholestane and coprostan, have definitely proved that cholic acid and coprosterol have the same carbon skeleton, and that cholic acid only differs in this respect from the latter by a loss of a  $\text{CH}(\text{CH}_3)_2$  group. Windaus has, we believe, gone so far as to suggest that the bile acids may be derived from the cholesterol in the organism. We know of no evidence that supports this suggestion.

For lack of a reliable method we did not attempt to determine the output of bile acids in the faeces of our infants, but from our sterol estimations it is obvious that no margin is available for the manufacture of bile acids from the

cholesterol of the diet. If the synthesis of cholesterol in the organism be accepted, we think it a more likely hypothesis that cholesterol and the bile acid are elaborated by collateral processes, according to requirements, rather than that cholesterol is first synthesised and then broken down into bile acid.

We take this opportunity of expressing our thanks to Dr. Jewsbury and the Matron of the Mothercraft Training Society, Trebovir Road, Earl's Court, Dr. Remington Hobbs of the Kensington Infirmary, Sister Nairne of the General Lying-in Hospital, York Road, Professor Lucas Keene and Dr. Innes Pearce of the Royal Free Hospital, and our own colleagues at St. George's Hospital, for the material and much valuable help in this work, and we also thank the Government Grant Committee of the Royal Society for help in defraying some of the cost of the work

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*The Action of Inorganic Salts on the Secretion of the Isolated  
Kidney.*

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In a recent paper by Starling and Verney (1) an attempt was made to study the functions of the kidney by the synthetic method. The organ cut out of the body was perfused with blood at the normal temperature and at any desired pressure from a heart-lung preparation. Under these conditions it was found to retain a considerable proportion at any rate of its fundamental functions. It gave a copious secretion of hypotonic urine, the amount varying with the blood pressure and with the dilution of the blood. By means of this preparation it seemed possible to build up the complete activities of the organ as they are found in the living body, by alteration of the conditions of the experiment, and thus to form an idea of the different factors involved in its normal activities. Thus, the low chloride content of the urine obtained from the perfused kidney shows that some factor is absent in the conditions of the experiment which is present in the kidney *in situ*. Reabsorption of chloride is evidently at its maximum, and we can raise the chloride percentage, either by restoring the factors which are normally present in the intact animal, or by paralysing all the activities of the tubules which tend to modify the composition of the glomerular filtrate. The method also allows us to use two kidneys at the same time, fed from the same heart-lung preparation. These two kidneys may be identical, so that one serves as a control of the other, or they may be from different dogs, or in different conditions resulting from varying periods of interruption of the circulation through them, or from the natural occurrence or experimental induction of nephritis. The use of two kidneys allows us to distinguish between effects in the urine due to changes in the circulating fluid and those which are due to alterations in the kidney itself. If the changes in the one kidney are localized, as apparently occurs in some forms of nephritis, we may use this method for attacking the question of the localization of function in the kidney.

The work of Hamburger (2) and his pupils on the perfusion of frogs' kidneys has shown the part played by the relative properties of Ca, K and  $\text{NaHCO}_3$



in the Ringer's fluids used in the perfusion. Our knowledge of the influence of the normal saline constituents of the blood on the secretion of the kidney *in situ* or fed with blood is extremely scanty. We propose in this paper to deal with the effect produced on the functions of the kidney by alterations in the amounts of K, Ca and phosphate ions present in the blood circulating through the kidney.

In a series of suggestive papers, Haldane (3) has dealt with the general effects of these salts on the urinary secretion in man, and he is inclined as a result of his investigations to ascribe the changes he observes in the urine rather to the alterations in the acidity of the tissues as a whole than to the chemical nature of the salt employed. A number of observers have drawn attention to the relationship which holds between the percentage of phosphates and the percentage of calcium. Thus Marriott and Howland (4) found that cases of nephritis may show an increase of serum phosphates and a decrease of calcium. Embden and Grafe (5) bring forward evidence for an antagonism between chlorides and phosphates in the urine during muscular exercise and the ingestion of phosphates. Freudenberg and Gyorgyi (6) found a high phosphate content and low calcium content in the serum in cases of infantile tetany. Kramer and Howland (7) in their investigation of experimental rickets in rats found a certain balance between calcium and phosphates in the food as well as in the serum of the animals. It is evidently impossible to understand the full bearing of these observations unless we know how the kidney functions are affected by alterations in the relative and absolute proportions of these ions in the blood serum.

The general methods employed in our experiments have been fully described in the paper by Starling and Verney (1). We have found that the period of delay at the beginning of the experiment, before secretion of urine, checked by the excision of the kidney and attachment to the heart-lung preparation, begins, is very much lessened and in some cases even abolished if care be taken to circulate the blood several times through the heart and lungs before allowing it to flow through the kidney. This is due to the fact pointed out by Eichholtz and Verney (8) that toxic substances with a vaso-tonic action make their appearance in defibrinated blood, but are removed more or less completely on the passage of the blood through the heart and lungs. In the absence of these substances we do not therefore get an initial constriction of the renal vessels, which may take a considerable time to pass off.

It is important to take the kidneys from young and healthy dogs. We have been struck in the course of our experiments with the frequency with which nephritis occurs, especially in older dogs, in the absence of any other signs of ill-health. In a number of our experiments we have used two kidneys at the same time to examine the differences in the results produced, either by differences in the duration of the time of cessation of the circulation through the kidneys, or by nephritis present naturally in one of the kidneys or artificially induced

by the previous injection of tartrates. In every case in which we had suspicions of the normality of the kidney, pieces of the organ were hardened and sections stained and examined microscopically. In all the experiments, hæmoglobin was estimated colorimetrically as HbCO, the first sample of blood being taken as the normal and designated by the number 100 per cent. In the course of experiment there is always a certain amount of concentration of the blood in consequence of the secretion of fluid by the kidneys. The concentration becomes much more marked when lung œdema begins. Most of the experiments are brought to an end by lung œdema. It must be remembered that in these experiments we maintained a pressure of about 110 mm. Hg at the level of the cannula in the renal artery, which involves a somewhat higher pressure in the aorta, and in some cases we have used a pressure as high as 140 mm. Hg at the kidney.

It is important also to observe the colour of the circulating blood. With commencing œdema of the lungs, even if this is not well marked, the rate at which the blood takes up oxygen in its passage through the lungs is markedly reduced. It has been shown previously that anoxæmia, if sufficiently marked, may cause a paralysis of the functions of the convoluted tubules, and it is important to be able to exclude the possibility of the intervention of this factor in the production of changes in the composition of the urinary secretion obtained. As soon, therefore, as the blood in any experiment showed signs of deficient oxygenation pure oxygen was used for the artificial respiration instead of air. This device in every case served to keep the blood well oxygenated until the lung œdema became sufficiently profuse to make any further continuation of the perfusion impossible.

Chlorides were estimated by Millard Smith's method (9), phosphates by Briggs's modification of the Bell d'Oisy method (10), potassium and calcium by the Kramer-Tisdall (11), urea by the urease method. In our tables of results urea phosphates are expressed in milligrammes per 100 c.c., potassium and calcium and phosphates in milligrammes K, Ca and P per 100 c.c. fluid, chlorides are expressed in grammes NaCl per 100 c.c., and the total solids of the serum in grammes per 100 c.c.

1. *The Influence of Potassium and Calcium on the Excretion of Chlorides in the Urine.*—A large number of experiments—26 in all—have shown that the actions of these two salts are closely linked together, so that although we started by investigating their effects separately, it will be advisable to reverse the order of our experiments and to consider first their joint effect on the kidney function. When these two salts in the proportion of 50 to 100 mgms.

of potassium to the same amount of calcium in the form of chlorides are added to the blood (about 900 to 1500 c. c.) circulating through the heart-lung preparation, no immediate effect is observed. And the same statement applies if the two salts are added within a short time, *i.e.*, within 20 minutes, one of the other. But in 20 to 30 minutes two results begin to be apparent; the most important and invariable of these, which is revealed only when the analyses have been made, is an increased concentration of the chlorides of the urine. As a rule there is also an increased flow of urine, but this is not so marked as the change in chloride content, nor is the amount of urine parallel to the concentration of chlorides. The typical effect of addition of these salts is shown in experiments 4 and 9, Charts I and II.

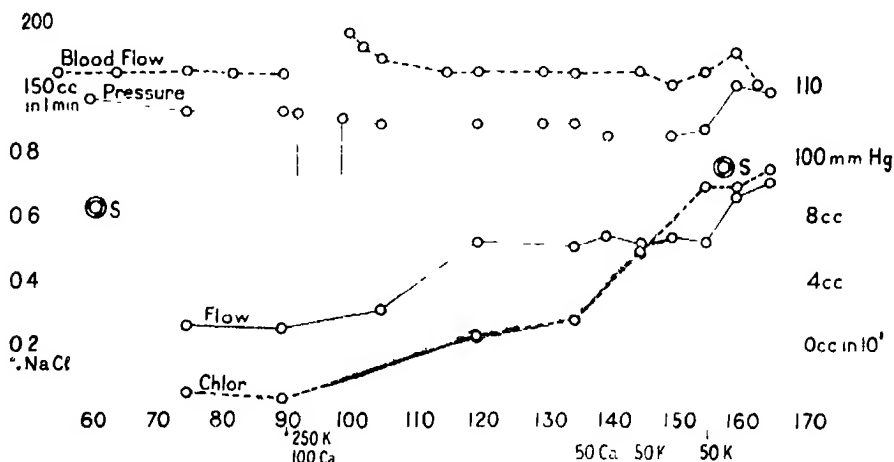


CHART I.—Exp. 4. Heart-lung-kidney. Kidney = 37 gms. Add 1 c.c. of adrenalin 1 : 100,000, 1.0 gm. urea. Connect circuits, 11 31. Urine flowing, 12.05.

In experiment 4, at the beginning of the experiment, the kidney was secreting 0.7 c. c. per 5 minutes with a NaCl content of 0.08 per cent. 90' after connecting the tubes to the kidney 250 mgms. K were added. This rather big dose stopped the heart, which immediately recovered after 100 mgms. Ca. The flow of urine rose immediately to 3.0 c.c. in 5 minutes and stayed at this level to 154'. In the meanwhile the chlorides rose steadily, attaining the figure of 0.27 per cent. at 134', 0.48 per cent. at 144', 0.68 per cent. at 154', and 0.74 per cent. at 164', further increased by additional doses of 50 mgms. Ca and 100 mgms. K. In the course of the experiment the chlorides in the blood serum had risen from 0.62 per cent. to 0.75 per cent., so that in this case the chloride concentration of the urine attained equality with that of the blood serum. In

Experiment 9, Chart II, the kidney from the outset was secreting copiously and was giving before any addition to the blood 8.5 c.c. of urine in 5 minutes

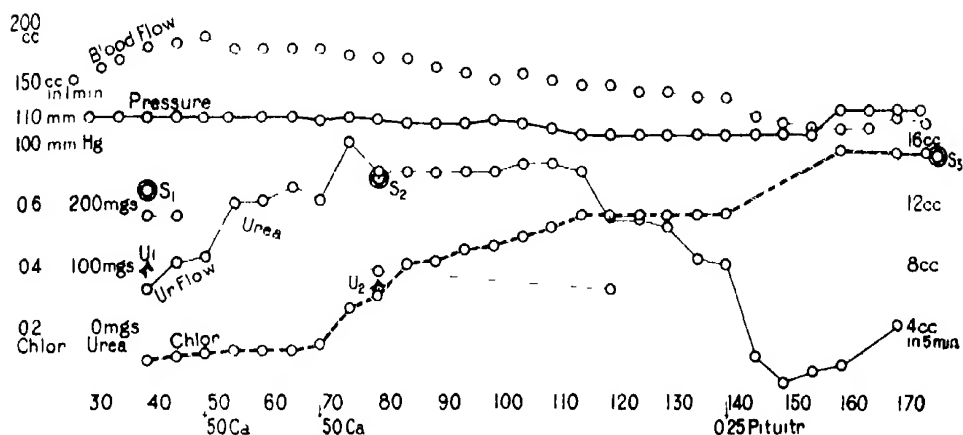


CHART II. --Exp 9. Heart-lung kidney Kidney = 48.5 gms. Add 1.0 c.c. adrenalin 1:100,000, 2.0 gms urea, 50 mgr Ca, 50 mgr K. Connect circuits 10.47 Urine flowing, 10.53.

At 11.36 50 mgms. Ca and 50 mgms. K were added, and this dose of the two salts was repeated at 11.56. The urinary flow rose by 12 o'clock to the colossal figure of 15.8 c.c. in 5 minutes, to sink soon to 14 c.c. in 5 minutes, at which figure it remained till 12.40. The urinary chlorides which at 11.35 amounted to 0.11 per cent, rose in 20 minutes to 0.14 per cent, and then more rapidly in 20 minutes to 0.45 per cent, and in another 20 minutes to 0.56 per cent, at which figure they remained constant to the end of the experiment. In this case the chloride percentage in the serum had risen from 0.64 per cent to 0.75 per cent, and the salts of the serum from 7.65 per cent. to 8.9 per cent., the great concentration of the serum being due to the large loss of water by the kidney, namely over 250 c.c. in the course of the experiment. The slow onset and later rapid rise of the chloride percentage induced by the addition of these two salts to the circulating blood is well shown in the accompanying chart (Chart 2). Out of 26 experiments in which the combined effect of these two salts was tried, we failed to obtain the above results on the chloride concentration in only one case. In this case the kidney, when examined histologically, showed signs of glomerular nephritis. We are not yet certain how far this condition was responsible for the absence of response to the Ca + K addition, since in other cases of natural and artificial nephritis we have obtained a

reaction to these two salts similar in all respects to that obtained with normal kidneys.

In order to determine what part is played by each of these salts in the production of the effects observed, we must consider the results of addition of each salt separately. The effects of an experiment on the influence of potassium alone are given in Chart III. In this case 50 mgms of potassium were added at 12.46, another 25 mgms at 1.06, another 25 mgms at 1.31, i.e., 100 mgms.

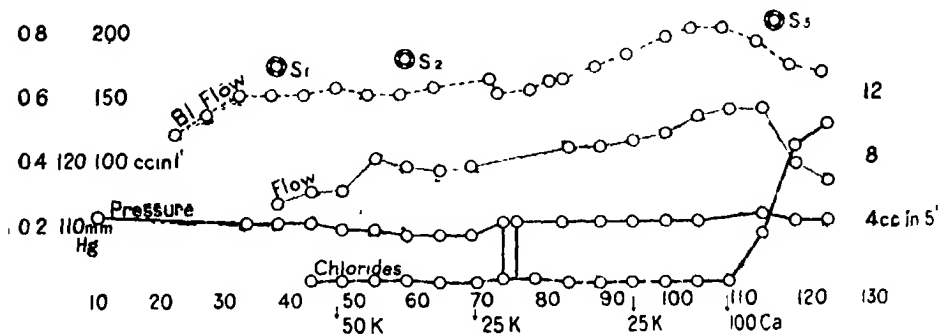


CHART III.—Exp 7. Heart-lung-kidney. Kidney — 32.5 gms. Add 1.0 c.c. adrenalin 1.100,000, 2.0 gms urea. Connect circuit 12.03. Urine flowing, 12.13

in all. After these additions there was a slight rise in the urine secreted, which might have occurred spontaneously. Before the first injection the urine was flowing at the rate of 6 c.c. per 5 minutes, and this gradually rose in the course of an hour to 10.5 c.c. The chlorides, which had amounted only to traces in the first specimens of urine, remained practically at this level, the early specimens giving 0.03 per cent. and the later specimens 0.01 per cent. The increase of potassium ions in the blood has therefore no influence in increasing the chloride concentration of the urine. On adding at the end of an hour 100 mgms Ca there was an immediate change in the composition of the urine, the chlorides being increased from 0.01 per cent. at 1.45 to 0.44 per cent. at 1.55.

On the other hand, if calcium be administered alone, without the simultaneous addition of potassium, there is either no effect on the chloride concentration of the urine or one which is slight and inconstant as compared with that produced by the addition of the two salts together. The results of two such experiments are shown in Table I. In experiment 1, where there was a very free flow of urine, the addition of 100 mgms. of Ca between 11.26 and 11.35, though causing a temporary augmentation of the urinary flow from 6.8 to 15 c.c. in 10 minutes, produced practically no change in the chloride concentration of the urine. This at 12 o'clock was 0.08 per cent. and rose in the course of an

Table I.

No of Exp	Mean Time	T ° C of Blood at Kidney	Bl Pr mm Hg at Kidney	Renal Blood Flow c.c / min	Serum			Urine				Remarks
					NaCl Per cent	Urea mgms Per cent	Potas. mgms Per cent	Flow c.c / 5 min	NaCl Per cent.	Urea mgms Per cent	Potas. mgms Per cent	
I	12 00	38 0	110	125	—	—	—	6 4	0 08	—	—	11 30 connect circuits
	12 15	38 5	110	143	0 79	—	—	6 5	0 06	—	—	
	12 25	36 5	110	143	—	—	—	6 8	0 07	—	—	12 26 50 mgms Ca
	12 34	36 5	110	158	—	—	—	15 0	0 07	—	—	12 35 50 mgms Ca
	12 50	36 5	110	158	0 82	—	—	10 3	0 07	—	—	
	1 05	36 5	80	150	—	—	—	5 8	0 07	—	—	
	1 10	36 5	110	150	—	—	—	14 4	0 13	—	—	
	1 25	36 5	80	136	—	—	—	6 6	0 11	—	—	
10	1.00	37 0	110	140	0 64	90	—	6 3	0 07	510	—	12 10 connect circuit, 50 mgms Ca
	1.05	37 0	110	143	—	—	—	6 6	0 07	—	—	
	1 10	37 0	110	140	—	—	—	6 7	0 07	390	—	1 10 50 mgms Ca
	1 15	36 9	110	136	—	—	—	6 2	0 07	—	—	1 15 50 mgms Ca
	1.20	36 8	114	120	—	—	—	5 9	0 07	—	—	
	1 30	36 8	118	100	—	—	—	4 2	0 17	—	—	Trace of blood in urine
	1 40	36 8	118	98	0 70	90	—	3 5	0 27	130	—	
	2 00	36 8	114	92	—	—	—	2 6	0 35	70	—	
	2 15	36 7	114	86	—	—	—	3 0	0 41	—	150	2 15-20 100 mgms K
	2 26	36 8	112	98	—	—	—	7 2	0 47	70	150	
	2 35	36 5	112	98	0 73	90	60	3 5	0 55	—	165	Weight of kidney — 37 0 gms

hour to 0.11 per cent. In the second experiment in this table there is no effect of calcium on the urinary flow, but there is some rise of chlorides towards the end of the experiment. We may conclude that calcium itself *may* lead to an increase of chlorides, but this increase is uncertain and is not comparable in extent with the increase which is induced when potassium is given at the same time. Moreover, we have the impression that excess of calcium in the absence of potassium in those doses which have been given in our experiments, has a poisonous effect on the isolated kidney, as is shown by the occurrence of traces of hæmoglobin in the urine.

It must be mentioned that the immediate effect of addition of calcium alone in small doses is a slight improvement of the contractile power of the heart, lasting about 2 minutes, and always some decrease in the flow of blood through the kidney if the dose of Ca is greater than 50 mgms. These effects are neutralized by simultaneous addition of potassium. If a still larger dose, *i.e.*, 200 mgms. of Ca, be given without the simultaneous addition of K, the heart may fibrillate. This has happened in several cases.

How are these results to be explained? It has been shown by Starling and Verney that in all cases urine secreted by the isolated kidney contains only small amounts of chlorides. The chloride concentration of the urine is not affected by a previous chloride-rich diet, nor by the addition of urea, of Ringer's fluid, of 2 per cent or even 5 per cent. NaCl to the circulating blood, or by the inhalation of CO<sub>2</sub>. A rise of blood pressure, which increases the rate of urinary flow, gives a correspondingly small increase in the chloride concentration, which they interpret as due to there being less time available for absorption. The chloride absorptive mechanism is therefore in these kidneys at its maximum activity. The only method they found, which could be described as physiological, which caused increase in the chloride content of the urine, was the addition of pituitrin in small doses to the circulating blood, though the chloride absorptive mechanism, together with all the other tubular mechanisms of secretion and absorption, might be thrown out of action by poisoning with cyanides or by extreme anoxæmia. The experiments quoted above show that these same results on chlorides may be attained by increasing simultaneously the concentration of the K and Ca ions in the circulating blood. On the other hand, whereas pituitrin diminishes the total urinary flow, the usual effect of K + Ca is to increase it. It might be thought that we are dealing here with a direct poisonous effect of the excess of these ions on the tubular absorptive mechanism, such as we get in anoxæmia or under the action of cyanides, and such an idea would seem to receive at first sight confirmation from the low urea concentration observed in Experiment 9, Chart II, at the height of the action of the K and Ca. A gradual decrease in the urea concentration is observed, however, in experiments where the chlorides remain low throughout, as in many of those quoted by Starling and Verney. The vitality of the tubular cells might also be presumed from the fact that they react to the injection of pituitrin with a slow further rise in the concentration of chlorides. Moreover, the effect of these two salts is at once abolished, as we shall see later, if the proportion of soluble ionic calcium in the circulating blood is diminished, as by the addition of phosphates to the blood. In many experiments, too, the ratio urine-urea/serum-urea was found quite high after the K + Ca had exerted their full influence on the chloride content of the urine. Thus in Experiment 22, (Chart V) the normal kidney gave a urea figure of 295, as against 114 in the serum at a time when under the influence of Ca + K the chloride content had risen from 0.03 to 0.3 per cent. and the total urinary flow from 4.6 to 10.6, and the tartrated kidney in the same experiment gave at the same time a urine/urea figure of 228 mgms. at a time when the NaCl concentration had risen from 0.02 to 0.4 per cent.

Moreover, the difference in the action of the salts, singly and together, would indicate that we are dealing here with a physiological or pharmacological rather than a toxic effect.

Salts which undergo dissociation may act in two definite ways. In one way, which is reversible, the reaction occurs immediately, so that there is a change in the physiological activity of the part affected within some seconds or parts of seconds. Examples of such effects we see in the action of calcium on the isolated frog's heart. The other mode of action is of slow development, leading in most cases to a change of structure and ending in death. This is the sort of action which occurs when lower organisms are exposed to unbalanced salt solutions, as in the experiments of Loeb and Wasteneys.

Our experiments show that the combined effect of calcium and potassium is of the chronic type, since it takes from half an hour to an hour before the new level of chloride reabsorption is reached. There is, however, a difference between the mode of action of the two salts involved, which will be evident from a consideration of the following statements:

1. K + Ca act after one hour
2. K alone or Ca alone have no or slight action
3. K alone has no action, but if one hour later Ca is added there is a change in the chloride concentration, which occurs immediately, *i.e.*, with ionic rapidity, so that the same effect is produced 10 minutes after the Ca addition as if it had been added with the potassium at the beginning of the experiment
4. If Ca has been added alone and one hour later K be added, there is a slow increase in the chloride concentration, as if both salts had been added when the K was added. This result, however, is inconstant.

It would seem, therefore, that these two ions play a different part in the production of the increased reabsorptive power of the tubules, the K ions being slowly built into some limiting membrane or active machinery of the cell, on which calcium can then act immediately. We may express this relation by saying that calcium acts on a background afforded by the presence of excess of potassium. It is, however, difficult to decide whether the action of these two salts is to be ascribed to a normalisation of a previously defective kidney, or to the stimulation of a physiological function in cells otherwise normal. We have already called attention to certain toxic properties of defibrinated blood, which are removed, at any rate to some extent, by the repeated passage through the heart-lung preparation. That defibrination itself does not produce abnormal response of the kidney is shown by the experiments of Starling and Verney (1), where normal NaCl figures were



obtained from the urine of a kidney *in situ* after total defibrination of the blood contained in the dog. It might, however, be objected that with the kidney in connection with the rest of the animal, detoxication would be more effective than when the blood passed simply through a heart-lung preparation.

An experiment to test this hypothesis was carried out as follows. A heart-lung preparation was made as usual. In a second dog the blood was rendered uncoagulable by defibrinating. Blood from the kidney was reinjected into the veins of this animal, while its carotid artery was bled into the venous reservoir of the heart-lung preparation. The blood thus passed through a whole dog as well as through the heart-lung-kidney preparation. The kidney, however, secreted a urine as poor in chlorides as in all the other experiments (0.08 per cent. to 0.11 per cent.)

Starling and Verney suggest that the primitive function of the kidney is to spare chlorides, as in the lower vertebrates, and that in mammals this function of reabsorption is held in check by hormones, such as pituitrin, produced in the tissues in accordance with their needs and with the actual state of the chloride reserves of the body. It may be that the effect of the  $K + Ca$  combination is to render sensitive the chloride reabsorbing cells, so that smaller amounts of pituitrin-like substances still present in the blood circulating through the kidney can exert their effects, or it may be that the action of these two salts together may replace some pituitrin-like hormone effect. As an analogy to this last suggestion we have the close relation of the calcium ion effect on the heart to that of sympathetic stimulation and of the potassium ion to that of vagus stimulation.

It might be thought that the slow rise of chloride concentration was connected in some way with the duration of the period of perfusion of the excised kidney. To test this suggestion, the kidney on the left side of a dog was excised and connected to the heart-lung apparatus. After one hour the right kidney, which had been kept alive by its normal circulation, was also excised and connected to the heart-lung preparation.  $Ca$  and  $K$  were now added to the circulating blood. The results of these experiments are shown in Chart IV. It will be seen that the latent period necessary for the onset of the greater chloride concentration, which we have found in every case, was the same in the second kidney as in the first. This shows that the latent period is not due to slow changes or interchanges taking place in the blood, or in the kidney as the result of perfusion, but to the necessity of time for the mixture of salts, *i.e.*, the potassium, to exercise its full effect on the absorptive mechanism of the kidney.

*Seat of action of these salts*—An examination of the various protocols shows that the increased chloride concentration of the urine is not dependent on

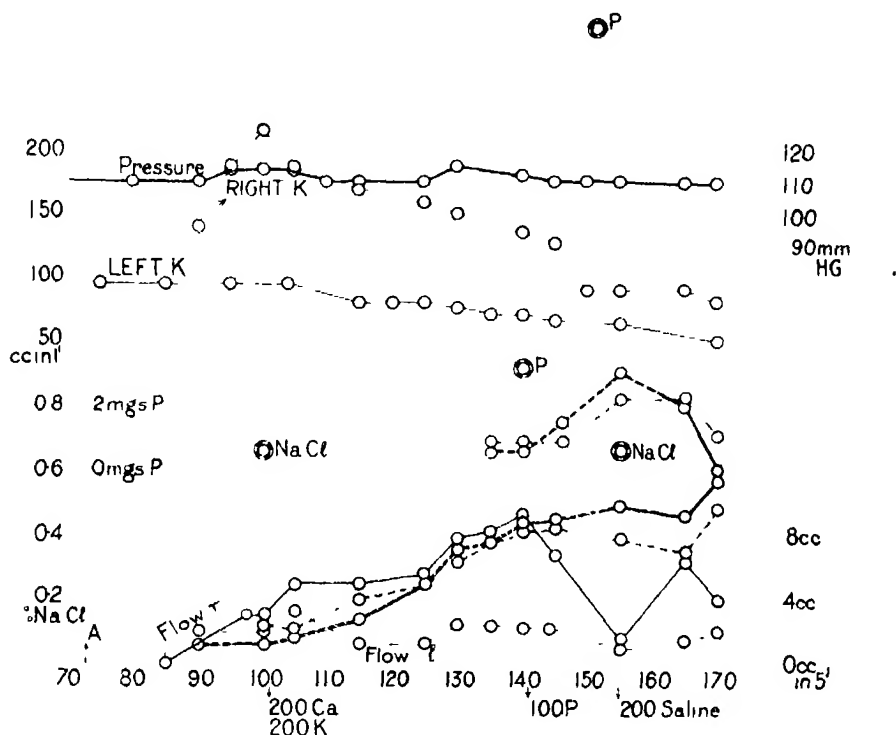


CHART IV—Exp 21 Heart-lung-kidney Left kidney connected 12 25 Animal survives.  
Right kidney connected 1.38. Add 3 0 gms urea, 1 0 cc adrenalin 1 100,000.  
Left kidney 46 0 gms, right kidney 45 0 gms  
A Right kidney connected

either the blood or the urinary flow. The chloride content may be rising while the urinary flow is increasing or diminishing, and is practically independent of the rate of blood flow or of the blood pressure. We may be certain, therefore, that the striking effects of the  $\text{Ca} + \text{K}$  ions are not due to any action on the glomerular filter, but must be ascribed to some change in the cells responsible for the chloride-absorbing function of the tubules.

*The effect of K + Ca on water output.*—The effect of these salts, separately or together, on the water output is illustrated by the Charts I-V. We may sum the results as follows:—

1. The effect of potassium salts alone is uncertain.
2. Calcium in small doses may lead to an increase of urinary flow. Larger

doses always cause constriction of the renal vessels and diminution of the urinary flow.

3. The same doses of both salts, which either have an uncertain effect or check the flow if given separately, lead, as a rule, to a large increase if given together.

4. Contrary to the comparatively stable chloride reabsorbing mechanism, the water-reabsorbing function of the kidney is very sensitive and readily affected by all sorts of influences

If the water-reabsorbing function is intact it behaves like the chloride reabsorbing function, *e g.* that on a certain background of potassium the excretion of water is a function of the calcium ion, and increase of calcium by adding it, or precipitation of calcium by means of phosphates, as shown later, lead to an increase or decrease in urinary flow. In this case effect on water output and on chloride excretion runs parallel

5 This last fact indicates that potassium and calcium affect directly or indirectly the water-reabsorbing mechanism of the tubules and not the filtration through the glomeruli

*Localisation of the calcium-potassium effect on the water output* --Generally speaking, there are five sets of conditions which in the whole animal may be effective in determining an increased flow of urine. These conditions would be changes in (a) tissue proteins, (b) serum proteins, (c) blood pressure or blood flow, (d) glomerular permeability. (e) reabsorption by tubules.

It has been suggested by Haldane (3) that the direct effect of calcium may be due to a change in the osmotic pressure of the tissue proteins, and this idea has been elaborately developed in a paper by Blum (12). In our experiments the bulk of the tissues have been eliminated, and we have to explain that in one experiment in the course of an hour 250 c.c. of urine were pressed out of 1,400 c.c. of blood under the influence of calcium + potassium. Under these conditions, any tissue influence must be insignificant

Ellinger (13) has suggested, on what seems to us totally inadequate grounds, that the effect of many drugs and salts is due to the direct effect of these drugs and salts on the osmotic pressure of the serum proteins, what he terms "*Wasseranziehungsvormogen*" or "*Quellungsdruck*." We hope to measure the magnitude of such changes in osmotic pressure of the proteins, if any, as are produced by the addition of the small quantities of salts used in our experiments.

But if Ellinger's explanation were correct the addition of calcium + potassium to the circulating blood should have the same result on two kidneys

connected with the system, whatever the state of the reabsorbing or filtering mechanism in the two kidneys. The experiments given in Charts IV and V show that when two kidneys are connected to the same heart-lung preparation the water output may vary in divergent directions in the two kidneys, so that the results obtained can hardly be ascribed to alterations in the osmotic pressure of the serum proteins. Any such change in protein osmotic pressure must be minimal under the influence of the small amounts of the salts we added, and it seems impossible to explain by such a change the pouring out of 250 c.c. of urine in the course of an hour from 1,400 c.c. of circulating blood.

Many observers have called attention to changes in the blood pressure and blood flow through the kidney under the action of calcium, and it has been suggested by Porgés and Pribram (14) that these vascular effects account for all the changes produced by calcium in the urinary secretion. We also have observed some changes in the same direction, but the effects on the heart and arteries play but a minor rôle in our experiments. We can bring about under the strictly controlled conditions of our experiments much larger changes in the blood flow through the kidney than those which occur after addition of calcium without producing any corresponding change in the urinary flow. There is no doubt that the results are due to the calcium ion acting on the kidney directly, and not to an action of the calcium on the circulatory mechanism.

Although it is impossible absolutely to exclude changes in the permeability of the glomerular membrane under the influence of these two salts, there can be no question that so far as chloride excretion is concerned we are dealing with an influence on the tubules. Our experiments show that the effect on chlorides and on urine flow is in most cases parallel. In some instances, as illustrated in Chart V, the parallelism is very close, the change of rate occurring with the same speed and reaching the same new level together. This fact would tend to confirm in some degree the hypothesis put forward by Starling and Verney namely, that the chloride-absorbing mechanism and the water-absorbing mechanism of the kidney are spatially separate, and that the Cl-absorbing mechanism is higher up in the tubule than the water-absorbing mechanism.

#### *The Influence of Phosphates.*

The urine obtained from the perfused kidney before any addition has been made to the circulating blood, when tested by Briggs's modification of the Bell d'Oisy method, usually contains a trace of inorganic phosphates in the

first one or two samples. The urine secreted later never contains a trace of phosphates. It was thought possible that alteration in the hydrogen ion concentration of the blood might lead to the secretion of phosphates. This was tested by artificial respiration with  $\text{CO}_2$  air mixtures, but was found not to be the case. Moreover, no phosphate output was obtained after the addition of calcium or pituitrin to the circulating blood.

If, however, sodium phosphate be added to the blood at the beginning of an experiment there is an excretion of phosphates in the urine. The phosphate excretion is at its highest in the next one or two samples and then gradually disappears. Thus in the experiment recorded in Table II phosphate corresponding to 50 mgms. P was added to the blood in the reservoir at 11.58. The figures obtained in successive ten minutes quantities of urine from 12.30 to 1.30 were as follows, in mgms. per 100 c.c. : 4.9, 6.2, 5.5, 3.9, 1.3, 0.8, 1.9, 1.1, a trace. The blood serum at 1 o'clock contained 5.1 mgms. P per 100 c.c., and the blood phosphates remained at this level throughout the experiment, showing that the waning of the excretion was not due to any disappearance of the phosphates from the blood. The question then arises whether the falling was due to a gradual increasing incapacity of the kidney to excrete phosphates as a result of the prolonged perfusion. The experiment was therefore repeated, using two kidneys instead of one, one of the kidneys having been removed directly from the living animal while the other had been left thirty-five minutes in the dead body cut off from the circulation before connection. The results of this experiment are shown in Chart IV. It will be seen from this table that the course of the excretion of phosphates was practically identical in the two kidneys, viz., a rise of the phosphate excretion shortly after the addition of phosphate to the circulating blood followed by a rapid decline. Nor could we ascribe the falling off of excretion to a gradual deterioration of the secretory functions of the kidney on the hypothesis that the sodium phosphate that had been added to the blood was at first being actively secreted by the tubules. The phosphate that appeared in the urine after injection of sodium phosphate behaved in every respect like a substance which after filtration through the glomerular membrane underwent a certain amount of reabsorption in the tubules. As a test of the occurrence of any specific constituent of the urine we may use changes in the percentage amount of the substance caused by alterations in the mechanical filtering conditions, *i.e.*, in the pressure of the blood supplied to the kidney. In the case of sodium chloride it was shown by Starling and Verney that increasing the urine by raising the blood pressure raised

Table II. Experiment 15 Heart-Lung-Kidney Kidney = 51.0 gms.  
Connect Tubes, 11.40. Urine flowing, 11.52.

Mean Time	T ° C of Blood at Kidney	Bl P mm Hg at Kidney	Renal Blood Flow c.c./min	Hb Per cent	Serum		Urine			Remarks
					NaCl Per cent	Phos. mgms Per cent	Flow c.c./5 min	NaCl Per cent	Phos mgms Per cent	
12 30	36.2	110	176	—	—	—	3.8	0.06	4.9	11 58 50 mgms P
12 40	36.0	112	176	—	—	—	4.1	0.07	6.2	100 mgms K
12 50	36.2	110	182	—	—	—	4.4	0.06	5.5	
1 00	36.2	110	182	100	0.78	5.1	4.8	0.06	3.9	1 11 pressure to 130
1 10	36.2	110	182	—	—	—	5.2	0.06	1.3	
1 15	36.0	130	214	—	—	—	10.6	0.04	0.8	1 20 pressure to 110
1 20	36.0	132	214	99	0.79	5.1	11.4	0.04	1.0	
1 25	36.0	110	200	—	—	—	6.4	0.04	1.1	
1 30	36.2	110	200	—	—	—	4.8	0.04	trace	
1 40	36.2	110	200	—	—	—	6.0	0.05	trace	

the chloride content in the urine corresponding with the increased flow, *i.e.*, the quicker the urinary flow through the tubules the more it tended to approximate in composition the glomerular filtrate. The same relation was found to hold good for the phosphates appearing in the urine after they had been added to the circulating blood. It will be seen in Table II that raising the blood pressure, say from 110 to 130, and doubling the output of urine, *i.e.*, from 5 to 10 c.c. in five minutes, resulted in an increase in the relative and absolute amount of the phosphates excreted. We are inclined, therefore, to regard the soluble phosphates—at any rate, after injection into the blood stream—as among those substances which pass through the glomerulus with the filtrate and are reabsorbed on their passage through the tubules.

On the other hand, we cannot explain the steady diminution which occurs in the excretion of phosphates as determined by alterations in the absorbing power of the tubules. The same rapid diminution in the excretion of phosphates occurs whatever the condition of the kidney we are dealing with, and is found to run a similar course when two kidneys are perfused simultaneously, one of which is “normal” and the other has been left in the dead body for some time, or is in a condition of nephritis as a result of the previous injection of tartrates into the animal. This relation will be apparent from the results given in Chart V, experiment 19.

We must therefore conclude that the falling of the phosphate excretion

after injection of these substances into the blood is due, not to a change in the excretory functions of the kidney, but to a gradual change occurring in the state in which the phosphate exists in the circulating blood.

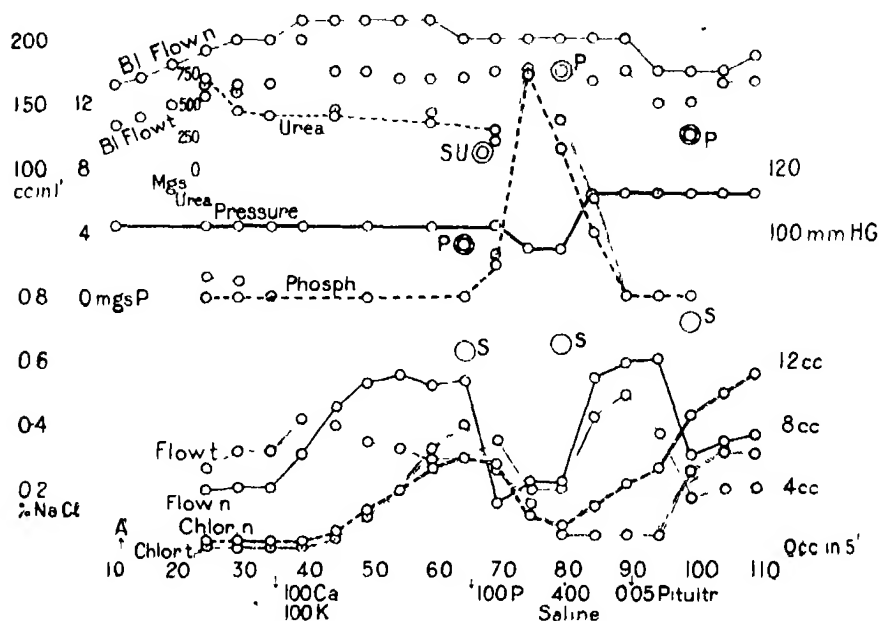


CHART V Exp. 22. Heart-lung normal kidney and tartaric kidney Dog, 12.5 kg. injected 1.5 gms per kg tartaric acid (dextro-rotatory) neutralized by means of sodium carbonate. Two days later left kidney connected to heart-lung 12.03 Urine flowing, 12.10 Normal kidney connected, 11.52. Urine flowing, 12.03 Add 3.0 gms urea, 1 cc, adrenalin 1:100,000 Weight of normal kidney = 49.0 Nephritic kidney = 42.0 gms.

A Second kidney connected

There is no question that this change in condition of the phosphates of the blood, which renders the kidneys impermeable to them, is much quickened if calcium be added to the blood shortly after the addition of phosphates. In this case, on the addition of calcium there is a steep fall in the phosphate content of the urine, instead of the gradual fall which is observed when phosphates alone have been added to the blood (Chart V), and with a large dose of calcium the excretion of phosphates is entirely abolished (see Table III).

Just as calcium stops the excretion of phosphates by the kidney, so does the addition of phosphate neutralize the effect of previous addition of calcium. On adding phosphates towards the end of a calcium + potassium experiment in an amount sufficient to raise the inorganic phosphate of the blood two or

Table III

No. of Exp	Mean time	T° C of Blood at kidney	Bl. Pr. mm Hg at Kidney	Renal Blood Flow.	Serum		Urine			Remarks
					NaCl Per cent	Phos. mgms Per cent	Flow c.c /5 min	NaCl Per cent.	Phos mgms Per cent	
18	11.56	37 0	110	200	0 67	3.5	3 5	0 05	trace	11 56 add 100 Ca 100 K 25 P
	12.01	37 0	110	214	—	—	6 7	0 09	trace	
	12.06	37 0	110	200	—	—	7.4	0 11	no	
	12.11	37 0	110	136	0 69	7 6	8 6	0 13	no	
16										12 00 add 100 Ca 100 K 50 P
	1 10	36 0	100	136	—	—	6 4	0 13	trace	12 27 Connect circuit
	1 20	36 0	100	150	0 63	7 2	6 7	0 11	trace	
	1 30	36 2	100	158	—	—	7 5	0 12	trace	

three times, no phosphate or only a small trace appears in the urine. On the other hand, the effect of calcium on the composition of the urine is removed. The chloride content, which has been raised as a result of the potassium + calcium effect, drops at once, and this drop in the chloride content is generally associated with a diminished urinary secretion. This effect is shown in Chart V. These results show that the calcium ion, either ordinarily present in the blood or added in the course of the experiment, slowly reacts with the added inorganic sodium phosphate and brings it into a modification to which the kidney is impermeable. This impermeability must be a function of the glomerular membrane. We are therefore justified in assuming that the phosphates, under the influence of calcium, are converted into a colloidal form, to which the glomerular membrane is impermeable, while remaining suspended and circulating in the blood. That the phosphate is bound in this way by the ionic calcium present in the blood is shown by the fact that the calcium excreted in the urine is diminished on the addition of phosphates to the blood (see Table IV). Thus there is a constant interaction between the phosphate ion and the calcium ion of the blood with a tendency to equilibrium, in which both ions are bound into a colloidal compound which circulates in the blood but does not pass through the glomerulus, nor is it excreted, under the conditions of our experiments, by the tubules.

As already mentioned, various authors have described an inverse ratio between the phosphates and chlorides of the urine in normal animals. In the



Table IV.

No of Exp.	Mean Time	Urine.				Remarks
		Flow c c /5 min	NaCl Per cent grms	Phosph Per cent mgms	Ca Per Cent mgms.	
23	1 37	4 6	0 18	trace	29	12 30. Add 150 Ca 150 K.
	2 07	4 7	0 24	trace	32	12 45. Tubes connected
	2 12	3 1	0 15	trace	—	2 07 100 mgms P
	2 17	3 1	0 11	2 6	26	
	2 22	3 0	0 10	1 8	10	
25	12 55	3 0	0 04	trace	50	12 30 Tubes connected
	1 00	—	—	—	—	1 00 Add 100 Ca 100 K
	1 15	6 0	0 19	trace	80	
	1 20	6 0	0 19	trace	118	
	1 31	—	—	—	—	1 21 Add 50 mgms P
	1 35	6 5	0 05	2 5	42	

case of the perfused kidney we have found such an inverse ratio and have been able to ascribe it to a binding of the calcium ion in the presence of excess of phosphates—the calcium ion, in the condition of these experiments, being essential for the excretion, or rather absence of reabsorption, of the chlorides in the urine. It would be interesting to inquire whether a similar causation is responsible for the conditions found in the intact animal.

The absence of phosphate excretion in the perfused kidney would seem to indicate that under the conditions of the experiment all phosphates are bound with calcium in a colloidal form, and are therefore not available for excretion. In view of this conclusion, we were considerably astonished with the result of perfusion of the kidney, before or after the addition of calcium, potassium and phosphates, with cyanided blood. The results of such experiments are shown in Chart VI. It will be seen that after the addition of cyanides to the blood, phosphates appear in the urine in almost the same concentration as they exist in the serum. They disappear again from the urine when the circulation of the cyanided blood is replaced by that of normal defibrinated blood. Starling and Verney assumed that cyanide affected only the excretory and reabsorbing mechanism of the tubules, and, so far as the chlorides in the urine are concerned, their results have been confirmed in our experiments. But the excretion of phosphates shows, we think without doubt, that cyanide is not without influence on the filtering mechanism of the glomerulus. It has

already been shown that if the perfusion of cyanided blood be continued for more than ten minutes protein begins to appear in the urine, showing an

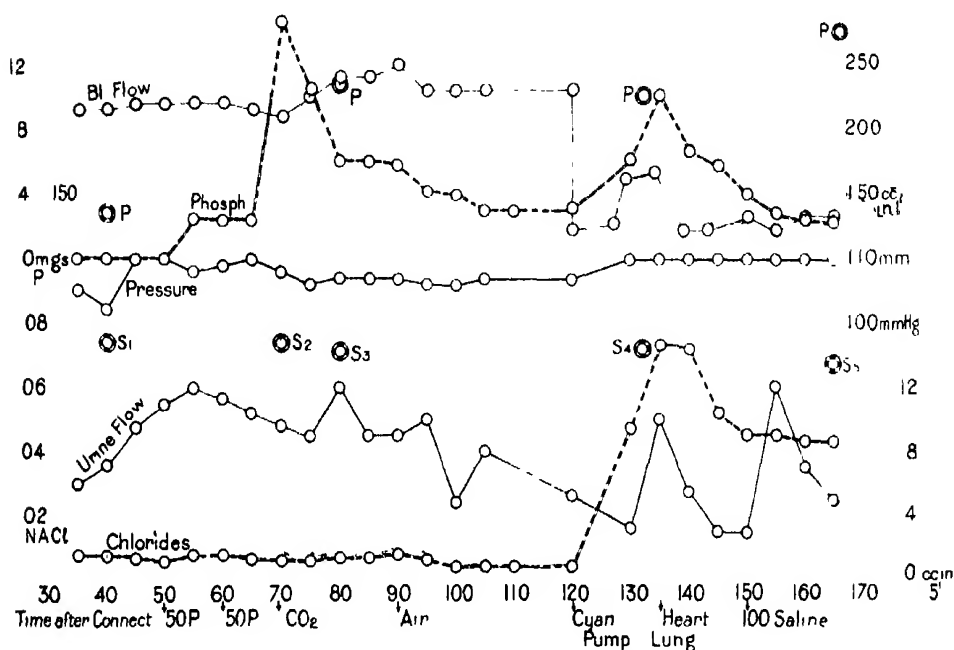


CHART VI.—Exp. 11 Heart-lung-kidney. Kidney — 34.0 gms. Connect circuit, 11.30  
Urine flowing, 11.41. Add 2.0 gms. urea, 1.0 c.c. adrenalin 1:100,000, 50 mgm  
calcium.

increased permeability of the glomerular membrane. This increase of permeability must begin some time before it betrays itself by allowing the escape of proteins through the membrane, and we are inclined to regard the results of our experiments as showing an increase in permeability of the glomerular membrane almost from the outset of the action of the cyanide, with the result that the glomerular membrane becomes permeable for the colloidal compound of calcium and phosphate ion, which we have assumed to be present in the circulating blood after the addition of phosphate alone, and still more after the addition of phosphate and calcium to the perfused blood. We regard, therefore, permeability to phosphates as indicating the first step of the changes that the glomerular membrane undergoes under the influence of cyanides. The result of these cyanide experiments shows that the retention of calcium in the blood circulating through the perfused kidney is not due to the calcium phosphate compound being bound up with the proteins, otherwise the appear-

ance of phosphates in the urine after cyaniding would be postponed to the time of appearance of coagulable proteins

It is difficult to avoid the conclusion that a great part of the phosphates present in normal serum are present as a non-ionised colloidal calcium compound, and not, as has generally been believed, in the form of the sodium salt of ortho-phosphoric acid. This is certainly the case under the conditions of our experiments, using defibrinated blood and a normal kidney, even before the addition of either calcium or phosphate to the blood. As has been already mentioned, under such conditions there is no excretion of phosphates by the kidney, but on cyaniding the kidney, phosphates pass into the urine in a concentration approximately equal to that in which they are found in the serum.

If this be true, the secretion of phosphates in normal urine must be due to processes of excretion by the tubular cells. As against this latter suggestion, it must be stated that we have obtained no evidence in the perfused kidney that this organ possesses the power of active excretion of phosphates similar to that which it possesses for urea, sulphates and certain colouring matters.

But we must bear in mind that under the conditions of the experiment the filtering processes of the kidney should be regarded as normal, and, as far as they are concerned, every fact points to the conclusion that the glomerulus does not take any part in the phosphate excretion.

It would appear that the phosphate secretion is more readily extinguished under the conditions of our experiments than the urea or sulphate excretory function.

### *Conclusions*

(1) Calcium, working on a background of potassium, leads to an increase of chloride excretion and water output, due to decreased reabsorption in the tubules. These salts, if given separately, have no definite effects.

(2) Inorganic phosphates decrease the output of water and chlorides by turning the calcium ion into a colloidal form.

(3) To this colloidal form of phosphates the glomerular membrane is impermeable. Cyanide increases the permeability of the glomerular membrane and allows the colloidal phosphates to appear in the urine.

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## Studies on Amphibian Metamorphosis.—II.

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### I.—Introduction.

In the following pages some experiments are recorded concerned with Amphibian metamorphosis. This process, apart from its intrinsic interest, is of general importance in two ways. In the first place it is one of the few morphogenetic processes which we can control, not only qualitatively, making it occur at will, but also quantitatively, as regards its rate. Secondly, it has been shown that metamorphosis in these forms is dependent chiefly upon the thyroid, and that metabolism is increased while it is taking place. It is thus possible to use it as an indicator of thyroid activity.

Various sets of experiments are here described. One concerns the effect of iodine and other agencies upon the Axolotl; another, utilizing the fact discovered by Swingle, that inorganic iodine will accelerate the metamorphosis of Anuran larvæ, endeavours to show how we can develop a method for testing what substances are antagonistic to the thyroid's metamorphic effect, what are auxiliary to it. Others deal with the pseudo-metamorphic effects produced by air-breathing upon the Axolotl, and with the possibility of keeping tadpoles under anæsthetics for a week and more, without interfering with the precocious metamorphosis produced by thyroid. Finally the failure of thyroid to produce specific effects on fin-regression in adult male newts in the breeding season is demonstrated and discussed.

## II — *Effects of Air-breathing on the Axolotl*

During the past three years I have kept a number of Axolotls of various sizes in shallow water, which was gradually decreased in amount, in order to see whether they would metamorphose, as should be the case according to the work of Marie v Chauvin (1876, 1885), and Boulenger (1913). In no single case has metamorphosis occurred, even when the animals have after some time been taken out of water and kept on damp moss (a treatment they will survive for at least several weeks). A similar failure was independently met with by Hogben (1922). Animals of all sizes, from full-grown adults to those 8 to 10 cm. long, were employed, the total number thus treated has been 16.

How is this failure to be explained? It is known (Swingle, 1923) that the New Mexican form of Axolotl shows a marked spontaneous tendency to metamorphosis, while this is extremely rare in the Old Mexican form, this shows that hereditary differences in regard to this tendency do occur. Further, some strains of Old Mexican axolotls have been bred for many generations in Europe without spontaneous metamorphosis ever occurring, whereas there are well-recorded instances in other strains; thus it is probable, on these grounds as well as *a priori*, that similar though less striking differences in this character occur in different strains of the Old Mexican race. Marie v Chauvin herself remarks that on seeing some of her animals come repeatedly to the surface, she judged the moment propitious for starting an experiment on the effects of air-breathing, to those who are acquainted with the habits of small Axolotls such as she used, comment is superfluous! She also makes it clear in her second paper that genetic differences as regards tendency to metamorphose exist within the species. In Boulenger's paper, no mention is made as to the

total number of animals tried, so that we are left in the dark as to the proportion of successes to failures \*

In view of the large number of failures, unrelieved by any success, which we have experienced although using methods both similar to and also more radical than those of earlier workers, I consider it probable that positive results can only be obtained in strains with a certain genetic tendency towards spontaneous metamorphosis. This supposition could be tested by trying whether by means of enforced air-breathing a greater proportion of New Mexican Axolotls could be made to transform than do so spontaneously—an experiment which I hope some American worker, who can easily obtain animals of this strain, will try

A remarkable morphogenetic effect of exposure to air was, however, found. During all the experiments carried out by me the fin along the dorsal mid-line of back and tail was left wholly out of the water

When this is the case, it falls over to one side, and after a certain time fuses with the skin of the back. The fusion becomes complete in the course of 2 to 4 weeks, not only is it impossible to pry the fin up with a needle, but no trace of the plane of union between the two sheets of epidermis is visible in sections. In these, however, the central mesodermal portion of the fin can still be seen lying parallel with the surface of the back and often curled over at its free edge. This is in complete contradiction to what occurs at metamorphosis (*e.g.* produced by thyroid or anterior-pituitary injections), when the fin remains vertical throughout, but gets smaller and smaller until it finally disappears altogether. (Plates 14 and 15, figs 1-3.)

Equally remarkable is the fact that when the animals are replaced in water the changes are reversed, the fin in the course of a week or so separating from the back and finally once more becoming upright. The fin-change at metamorphosis, on the other hand, is only reversible up to a certain stage, the final part of the process being irreversible

When the fin is well fused, the external appearance of the back of the animal is very like that of a metamorphosed specimen. It is only sections which reveal the essential difference. It is interesting to find the same general morphological result obtained in two such different ways. It is also interesting to note that the method adopted as *direct* response to change of environment is not that found in artificial metamorphosis in the Axolotl or in normal metamorphosis

\* Mr. Boulenger has since kindly informed me that he failed consistently with full-grown specimens: of 11 small-medium specimens in his final experiment, 5 died and 6 metamorphosed

elongated hind-limb buds showing the first traces of differentiation into main regions, can be kept in narcosis for 8 to 10 days with comparatively low mortality in the appropriate dilution of urethane. The dilution varies with different strains of tadpoles, but is in the neighbourhood of 1/300.

Tadpoles fed once or twice on fresh thyroid (in this case ox gland), or immersed for 1 to 2 days in a solution of thyroid made from desiccated thyroid tablets (Armour's)—strength of solution used 1/600 to 1/3600; filtered (or, better, centrifuged and decanted)—will, as is well-known, begin to metamorphose precociously, distinct tail-resorption and hind-limb differentiation are visible in 4 to 6 days at about 16°, and the fore-limbs appear in 8 to 12 days. If a number of tadpoles treated in either of these two ways are placed in urethane, the rest being retained in tap-water as controls, it is found that both sets undergo the sequence of metamorphic changes in the same way. While careful quantitative experiments would be needed to determine whether narcosis had no delaying action at all on the process, it can be asserted that if a difference in rate exists it is very slight. The only marked differences between the two lots are (I) that the mortality of thyroid-treated tadpoles in urethane is greater than that of those treated either with thyroid alone or with urethane alone, and that many of them become œdematous.

There are different degrees of narcosis with urethane: the tadpoles may remain quite insensitive to stimuli at the bottom of the dish (or rarely floating), or they may respond to strong or to weak tactile stimuli, while remaining motionless if undisturbed, not responding to the slight vibrations which make normal specimens career round the container. Even in complete narcosis, respiratory movements are of course maintained. It appears to make no difference—except to the mortality rate—what degree of narcosis obtains in these experiments.

Another series of experiments was made in which tadpoles, either from stock or straight from a previous period of narcosis, were put into similar thyroid solutions, but made up in the appropriate strength of urethane. In these they remained, of course in narcosis, for from 2 to 3 days. On being placed in water, these also metamorphosed normally.

These preliminary experiments thus show that in tadpoles both the absorption of the thyroid "hormone" from solution, and its subsequent action upon metamorphosis, can take place while the animals are in complete narcosis.

Thus the metamorphic effects of thyroid, involving in different regions of the body increased growth-rate, decreased growth-rate, increased rate of differentiation, and retrogression (dedifferentiation), can take place independently

of the higher centres of the central nervous system and of voluntary movement. It is quite possible that these factors may exert a small secondary effect upon its *rate*, but it is thus made probable that the place of attack of the thyroid substance must be either in the nerve-endings of the autonomic system, or in the tissues themselves.

I may add that, after a little previous work to determine the correct strength of urethane which will keep the tadpoles narcotised and yet not kill them in the course of the needful 6 or 8 days, the procedure is extremely simple, and that the effect made on the imagination by the spectacle of differentiation proceeding normally in the insensible organism makes the experiment a good one for purposes of demonstration. The shortening of the tail, the growth and differentiation of the limbs, and the alteration of body-shape are marked within a week at ordinary temperatures, although *complete* metamorphosis takes longer, and is usually interrupted by death in such young tadpoles shortly before the time for emergence from the water. A brief preliminary note on this subject was given at the International Conference of Physiology at Edinburgh in 1923 (Huxley, 1923)

Since then, working with the Barcroft differential apparatus, I have found that oxygen consumption of tadpoles treated for 24 hours with moderately strong thyroid solutions 4 to 6 days previously, is increased about 100 per cent. per gram body-weight (about 50 per cent. per individual animal); and that urethane narcosis depresses the oxygen consumption of both control and thyroid-treated tadpoles about 40 per cent. These results are approximate and preliminary only; and I hope to extend them considerably during the coming year. It is important to note, however, that the considerable depression of oxygen consumption caused by urethane in thyroid-treated tadpoles does not prevent metamorphosis from proceeding at the normal rate.

I should like here to express my thanks to Dr. Barcroft for the hospitality of his laboratory and for much kind help and advice.

I append the details of some of the experiments. According to Dixon (*Principles of Pharmacology*) tadpoles can be narcotised with urethane in solutions of 1/25000 (0·04 grm. per litre). This strength was tried, but found to be wholly inefficacious. A series was then made up with the following results :—



Table I.

Strength.	Minutes needed to narcosis	Minutes needed to stop circulation	Remarks.
1/20	1 to 2	10 to 12	—
1/40	3 to 4	15	—
1/100	6 to 7	Much reduced after 16 m	Recovery on return to water after 16 m.
1/200	10	A few slowed in 25 m    Some still alive, 16 hrs	Recovery on return to water
1/3200	No effect	No effect	—

Repetition with a fresher solution and other strengths showed that dilutions of 1/450 and over induced temporary sluggishness which wore off in 48 hours. 1/400 was similar, but might induce total immobility in some. 1/200 was fatal to some but not to others in 48 hours.

A third experiment showed the following results of 24 hours' exposure to various dilutions —

1/400 all sluggish ; 1/350 very sluggish ; 1/300 mostly non-motile ; 1/250 all non-motile, no deaths in any dilution.

An experiment with urethane and thyroid was then started. The tadpoles were all selected with hind-limbs showing the main limb-divisions, while a few had the first indication of digits. The thyroid solutions were made up with Armour's 5-grain tablets. None of the animals were fed during the experiment. The results are given in tabular form (see Table II).

We notice the great mortality caused by thyroid-plus-urethane as against either alone, and the œdema in the (T.F. + Ur) specimens. The circulation in urethane specimens previously treated with thyroid appeared better than in those not so treated. In all urethane-treated specimens, notable amounts of fæces were extruded, but this was much more marked in those previously thyroid-treated, in which a string of fæces might extend to beyond the tail tip.

The specimens in 1/230 urethane did not react when touched ; some of those in 1/250 urethane did so slightly.

A second similar experiment was executed thus : 26 half-grown tadpoles were placed in 1/800 thyroid solution (Armour's tablets) for 52 hours, and thence transferred to 1/300 urethane. (May 27.)

After 1 day, 12 were dead. 2 showed spontaneous movements ; the remaining 12 were motionless, but 3 reacted to stimulation.

Table II.

Designation.	Treatment.	May 19	May 21.	May 22	May 23.	May 25.
Controls	Tapwater throughout—10 specs	—	No change	No change	No change	No change.
TS	May 15, desiccated thyroid solution— 1/400 24 hours, 1/800 further 24 hours	—	All, marked metamorphic signs, digits well defined.	At May 21, a little more advanced. Tail resorption definitely started	Now not so advanced as T.F.	All alive, 2 with L. fore-limb, little tail change.
TS + Ur		3 dead.	All, marked metamorphic signs, digits defined	One dead, more advanced than any TS. One alive, as advanced as any TS	Last one died, with L. fore-limb protruded	—
Ur	To 1/230 urethane, May 17—9 specs	—	No change	No change	No metamorphic changes. One dead, two with moderate, five with good circulation	One dead, six alive, moderate to fair circulation
T.F.	Meal of fresh ox-thyroid, May 16 and May 18; also some squeezed into water.	—	Body of all fiddle-shaped, none oedematous.	Six with tail resorption started; digits showing	Three dead, five alive, two with both fore-limbs showing	Five alive, three with both and one with L. fore-limb. Two with shorter tail than trunk.
T.F. + Ur		1 dead	Body of all fiddle-shaped. Most oedematous.	Five dead, two alive, metamorphosis as advanced as T.F. Three with oedema	Both marked dead, tail resorption, but no fore-limbs. One with oedema	—

After 3 days, 1 more was dead, 3 showed spontaneous movements, and of the remaining motionless 9, all but 1 reacted to stimulation. This condition, in which most remained motionless but reacted to stimuli, was maintained. Finally on June 7 (13 days) all were found dead in advanced stages of metamorphosis, with the tail less than the trunk in length.

The next experiments were designed to see whether thyroid could act equally well when the thyroid-treatment was given under narcosis.

June 4, 4 large specimens, with hind-limb digits differentiated, were placed in 1/300 urethane. On June 6 they were transferred to 1/3000 thyroid in 1/300 urethane, and after 18 hours once more replaced in 1/300 urethane. They were all immobile, but reacted slightly to mechanical stimuli (touching with needle). After 6 days, all showed metamorphic signs, 1 with 1 forelimb apparent, 1 with tail length reduced to trunk length. 3 were lying on their backs, 1 floating. June 13 (7 days), similar but more advanced, 1 dead. Thus the taking up of thyroid as well as its subsequent metamorphic action can occur under narcosis.

May 28, a batch of half-grown tadpoles was divided into two lots. One (B) was exposed to 1/3000 thyroid solution in tap-water, the other (A) to 1/3000 thyroid solution in 1/330 urethane. After 3 days both were transferred to tap-water. The metamorphosis of those exposed to thyroid under narcosis was no further advanced than that of the others.

Of the 34 specimens in (A) after 5 hours in the thyroid and urethane, 30 were completely narcotised (irresponsive to stimuli), 2 responded to stimuli, 2 showed spontaneous movement. The same sort of proportion was found after 24 hours. After 3 days, however, two were immobile but responded to stimuli, and 22 showed spontaneous movement (the rest were dead). This recovery from the effects of urethane after long exposure to border-line strengths is frequent, but I have never seen it so marked as here. Very possibly the increased basal metabolism caused by thyroid is responsible in this case.

In another experiment, 35 smallish specimens were exposed to 1/3200 thyroid solution in 1/320 urethane. After 36 hours, 3 only were alive. These were placed in tap-water, and in 10 to 13 days from the start showed advanced metamorphic signs (tail reduced to about length of trunk).

During the urethane treatment, all were totally insensible to stimulation.

Another experiment was tried to see whether tadpoles previously treated with thyroid became narcotised in urethane as quickly as control specimens. The thyroid-treated specimens had been immersed in 1/800 thyroid solution

for 24 hours 4 days previously, and were showing slight metamorphic signs. The strengths of urethane were as follows, both for thyroid-treated and control specimens —

A	B.	C.	D.	E.	F.	G.	H.	J.
1/300	1/240	1/280	1/320	1/360	1/400	1/440	1/480	1/520

The animals were tested by touching them with a needle at frequent intervals

A to E were tested during the first 10 minutes, D to J from 10 to 30 minutes. In all cases, the controls went into narcosis slightly sooner than the thyroid-treated. After 1 hour, both thyroid-treated and controls were all completely narcotised in A to D; in E to J the thyroid-treated were less deeply narcotised.

In 1½ hours, all of both lots in E were in complete narcosis. After 18 hours, some recovery was noted in both lots in E. At 60 hours, however, the thyroid-treated in F to H were in deeper narcosis than the controls, they also showed a far higher mortality.

Thus previous thyroid-treatment of tadpoles makes entry into urethane-narcosis less rapid, but tends to prevent later recovery (as well as increasing mortality).

Table III.

		Spontaneous		React to Stimuli.	Complete narcosis.		Dead.	(Edema- tous )	
		Total	Movement		With circulation	Without circulation			
2 days	A	Th	17	—	—	3	14	—	(7)
		Cont	11	—	—	3	8	—	—
	B	Th.	18	—	2	9	7	—	(3)
		Cont.	16	—	9	5	2	—	(2)
	C	Th	17	1	7	3	—	6	(8)
		Cont	13	1	12	—	—	—	—
5 days	A	Th	17	—	—	—	—	17	—
		Cont.	11	—	—	—	—	11	—
	B	Th.	18	—	2	1	—	15	—
		Cont.	16	—	6	2	—	8	—
	C	Th.	17	—	2	—	—	15	—
		Cont.	13	6	7	—	—	—	—

A second experiment was tried along similar lines. The tadpoles here were slightly more advanced in metamorphosis than in the previous experiment: only 3 strengths of urethane were used—A, 1/200; B, 1/330; C, 1/440.

It was found that the heavy death-rate, the stoppage of circulation, the cedema, and the lack of recovery, were manifested at from 36 to 48 hours by the thyroid-treated specimens in all 3 strengths. Almost all the thyroid-treated were dead after 5 days. The results may be briefly summed up (see Table III).

#### IV.—*Failure of Iodine and Oxygen to induce Metamorphosis in the Axolotl*

*Expt. 1.*—It is well known that inorganic iodine, whether in solution or administered in the food, will cause precocious metamorphosis in Anuran larvæ (Swingle, 1918). Recently, however, it was found (Huxley and Hogben, 1922) in the neotenus Axolotl, that although it can be readily and invariably transformed into the Amblystoma form by thyroid-feeding (Huxley and Hogben, *ibid.*; Jensen, 1916, Kaufman, 1918), yet the administration of iodine in the food did not cause metamorphosis, even after a considerable time (8 to 10 weeks). The work of McCarrison (1917) and Adler (1916) suggested that the available oxygen supply might be concerned with thyroid activity. Accordingly the following experiment was designed to test whether iodine-administration in an atmosphere of oxygen was more efficient in promoting Axolotl metamorphosis than in air.

A saturated solution of iodine was obtained by shaking up the crystals with tap-water. The iodine content of this solution was estimated for me by Mr. Walden and found to be 0.076 mg iodine per litre. Dilutions of this were made as follows: 1/25, 1/50, 1/100 and 1/250. It was found as a result of preliminary experiments that the 1/25 solution killed Axolotls (2 specimens) within 3 days. The 1/50 solution killed 1 specimen in 4 days. The 1/100 solution was the strongest used in the main experiment.

All the animals used were small-medium immature specimens, varying from over 70 to under 90 mm. in length.

A broad shallow bell-jar was fitted with clamped inlet and outlet tubes, and filled with oxygen from a cylinder after inversion over a tray containing water. Four vessels with one Axolotl each were placed in its contained atmosphere of oxygen. Four similar vessels exposed to air were employed as controls. The arrangement of the vessels were as follows: A, B, C, D in O<sub>2</sub>; F, G, H, I in air. A and I in water; B and F in 1/100 iodine solution; C and G in 1/250 iodine solution; D and H in water, iodine-fed. All were fed on ox muscle.

For feeding, the iodine was dusted on to thin slices of meat which were then rolled up. Each vessel contained 250 c.c of liquid ; the depth of liquid was just over 30 mm. The animals were fed and the oxygen atmosphere renewed twice a week. I have to thank Dr. C. G. Douglas, F.R.S., for advice as to the experiment, and for determinations which showed that the percentage of oxygen under the bell-jar remained as high as 90 per cent. after 4 days. The results are given in tabular form (Table IV).

They may be summarised as follows. Inorganic iodine, whether in solution or in the food, does not cause metamorphosis in Axolotls within a period of 4 months, whether the animals are exposed to an atmosphere of air or of oxygen.

On the other hand, many of the changes associated with the early stages of metamorphosis do occur as a result of the treatment, these are most marked in 1/100 iodine solution, less so when iodine is given with the food, and very slight in 1/250 iodine solution ; and they are increased by exposure to an atmosphere of oxygen.

Specimen B unfortunately died, possibly by drowning, after 96 days. by this time it had made very considerable progress in metamorphosis. The final moult, however, which is characteristic of the last irreversible steps in the process, had apparently not yet occurred ; until this takes place, and the animal leaves the water voluntarily, we are not justified in speaking of complete metamorphosis. On the other hand, that both iodine (above a certain concentration) and oxygen do exert an effect which is of the same general sort as that which is operating at true metamorphosis, appears clear from the following facts.

During metamorphosis caused by thyroid, growth is much retarded, or there may even be a decrease in size (Huxley and Hogben, Boulenger, Kaufmann).

The changes which I have spoken of as metamorphic in character are this retardation in growth, a reduction in the gills and fin, the protrusion of the eyeballs, the alteration in the shape of the head, and the lightening in colour. As the last column but one of the table shows, these go together in what we may call the incomplete metamorphosis caused by iodine (as well as in the complete metamorphosis caused by thyroid). The last column shows that there is a marked correlation between the extent of these changes and the amount of retardation of growth. Furthermore, although in A, C, G, H and I these changes are absent, or so slight as to be obviously reversible and hardly to merit the term metamorphic, yet there is still a gradation both in them and in size. G and I are the largest in size, the darkest in colour, and possess the finest



gills. C and A are receiving the same iodine treatment as G and I, but have been exposed to oxygen; they are markedly smaller, and lighter, and their gills, though still within the bounds of normal variation, are distinctly less well-developed. The gills of H are in the same condition, but its fins and eyes show slight changes, its size is smaller.

We are apparently justified in saying (1) that 1/250 iodine solution exerts an almost negligible effect. (2) That exposure to an atmosphere of oxygen inhibits the growth-rate, the fullest development of the gills and the dark colour, but produces no effect upon the fin or eyes. (3) That iodine-feeding or exposure to 1/100 iodine solution alone produces very faint metamorphic effects, upon growth-rate, gills, eyes, fins and colour, but is not capable of causing complete metamorphosis. The effects of the 1/100 solution are the stronger. (4) That the effects of the 1/100 iodine solution and of iodine-feeding are distinctly enhanced by an exposure to an atmosphere of oxygen. (5) That exposure to 1/100 iodine solution in an atmosphere of oxygen may bring about marked but not complete metamorphic changes in 3 months. The question as to whether iodine-plus-oxygen could ever bring about complete metamorphosis in Axolotls must for the present be left unsettled.

*Expt. 2*—Two small medium-sized Axolotls, one white and one black, were placed in small vessels tilted so that the animals were about half covered with water when at the lowest point of the dish. They were then placed in an atmosphere of oxygen. On 17.5.22 it was noted that the white Axolotl was considerably more pigmented than when put in  $O_2$ . This excess pigmentation remained until 5.6.22, when the animal died, after moulting partially on 3.6.22.

The black specimen moulted partially on 20.5.22, and again on 10.6.22 and 24.6.22. On 1.7.22 it was removed to air, and placed on damp moss.

#### V.—*Experiments with Iodine and Thyroid in conjunction with other agents in Tadpoles*

As there thus seems to be evidence of a reinforcing effect of oxygen and iodine in the causation of metamorphosis, it was resolved to test the matter upon frog tadpoles. Here, as Swingle (1918, 1919) has shown, a quantitative relation can be obtained between the rapidity of metamorphosis and the strength of iodine solution in which the animals are immersed.

*Expt. 3*.—Started 10.4.22. Medium-sized tadpoles (about 20 mm.).

The following vessels were prepared, with 20 tadpoles in each. A to D, in oxygen; E to H, in air; A and E with strong, B and F with moderate, C and G



with weak iodine, D and H control (water). Tap-water was used throughout. The dishes were changed three times a week.

The amount of saturated iodine solution used for 200 c.c. was at first : A and E, 4 drops, B and F, 2 drops ; C and G, 1 drop. Later this was increased (15.4.22) to 8, 6, and 4 drops respectively, and C was divided into C<sub>1</sub> with 4 drops and C<sub>2</sub> with 2 drops. Since many deaths had occurred in the oxygen dishes by 15.4.22, the tadpoles in these dishes were made up from stock on this date to their original numbers. The drops were given with a Dreyer pipette.

No deaths occurred in the controls throughout, but on 21.4.22 the great majority of the oxygen specimens had again died, and the experiment was closed. The air specimens grew rapidly, and retained the normal ovoid body shape. Those in oxygen, however, not only remained without growing, but became very thin, the body acquiring the fiddle-shape which characterizes thyroid metamorphosis. Before death they often became markedly œdematous.

*Expt. 4*—To corroborate the last experiment, the air specimens were divided into two lots on 21.4.22, and one lot (A to D) placed in oxygen, while the rest were continued in air (E to H). They now averaged 2 or 3 mm. more in length than on April 10. The iodine dosage was increased as follows. A and E, 14 drops ; B and F, 11 drops ; C and G, 8 drops per 200 c.c. The dosage was increased on 3.5.22 to 24, 20 and 16 drops respectively ; the experiment was closed on 10.6.22. Thirty-one of the 40 oxygen specimens had died, but only 10 of the air specimens.

The same differences in rate in growth and in body-shape were noted as before ; but in addition an effect on the growth of the limb-buds was found, those of the specimens in oxygen growing *less* rapidly than those of the specimens in air. It is clear that the increased oxygen tension has a markedly deleterious effect upon the tadpoles' growth and general health. In spite of the resemblance of the body-shape of the oxygen specimens to those that have been fed on thyroid, the growth of the limbs induced by iodine is also retarded, and retardation of limb-growth appears to be greater than was to be expected from the retardation of general growth. Presumably the higher oxygen tension is directly poisonous to the tadpoles, as it is known to be to mammalian tissue (Haldane, 1922, p. 356).

*Expt. 5*.—An experiment was now started (10.5.22) to test the effect of an atmosphere of oxygen on metamorphosis induced by thyroid-feeding.

Six dishes containing 200 c.c. of water were prepared as follows : A to C in oxygen, D to F in air. A and D medium tadpoles (17 to 20 mm. long), B, C, E

and F, smaller tadpoles (14 to 16 mm long) A, B, D and E were fed on thyroid, C and F on meat.

The experiment was closed on 22.5.22. Those in oxygen were then smaller than those in air. Distinct metamorphic changes were seen on 19.5.22. The thyroid-fed specimens in air were then, and continued later to be, more advanced in metamorphosis than those in oxygen. For example, on 20.5.22 in D three showed partial tail resorption, one had the left fore-limb visible. No fore-limbs or tail-shortening had occurred in A.

On 22.5.22 of 9 in D 8 had died during metamorphosis, 6 of these showed the left fore limb. In A on the same date, 4 showed partial tail-resorption, but none had fore-limbs. It was noteworthy that none had died in A. This corroborates Jarisch (1920), who found that an atmosphere of oxygen prevented most of the mortality that commonly occurs when tadpoles are very precociously metamorphosed by means of thyroid-feeding. Thus oxygen, although inimical to general health, is beneficial at the critical stages of precocious metamorphosis, presumably because oxygen consumption is then high.

The smaller specimens in B and E were, as might be expected, less advanced metamorphically than those in A and D on corresponding dates. More died in B than in A, and the survivors were thinner. Two became œdematous in B, none in A. *I.e.*, smaller tadpoles, as we have previously seen, suffer more from exposure to oxygen than do larger ones. As before, B (oxygen) was less advanced in metamorphosis than E (air). Thus exposure to pure oxygen retards even the rapid limb-formation induced by thyroid diet.

*Expt. 6*—A further experiment corroborative of the last was started on 27.5.22. Fifteen tadpoles were placed in each of two dishes and fed on thyroid, those in one dish were placed in oxygen. Three of the oxygen and one of the air specimens died before 1.6.22. After this, rapid metamorphic changes ensued, and all the animals died (as often occurs after thyroid feeding) before 3.6.22. But whereas 7 of the oxygen specimens were still alive on 2.6.22, all the air specimens were dead on this date. On 31.5.22 the metamorphosis of the air specimens was further advanced than that of the oxygen specimens.

Examination of those that died on 2.6.22 and 3.6.22 during metamorphosis gave the following result:—

Table V.

	Fore-limbs	Oxygen (3.6.22)	Air (2.6.22)
Well developed		4	5
Moderately developed		3	1
Slightly	..	3	5
Not	.	2	3

All showed marked tail-resorption

Thus the stage reached at death was about the same in the two cases, but those in oxygen took somewhat longer to reach it than those in air. The metamorphosis was noticeably more rapid than in the previous experiment (7 as against 12 days). This was probably due partly to the employment of larger tadpoles, possibly also to differences in the thyroid used. In any case the rapidity of the process tended to obscure the delay in metamorphosis caused by oxygen, although this was still distinct.

*Expt. 7.*—Simultaneously another experiment, with iodine and oxygen, was started on 27.5.22. Two sets of vessels with strong and weak iodine and tap-water were prepared. Fifteen tadpoles were placed in each dish. One set of vessels (B to D) was placed in oxygen, the other (F to H) in air.

Distinct differences in size and limb-development were noted by 3.5.22. The size of all the oxygen specimens (B to D) was markedly less than those in air. Within either series, the specimens in water were the largest, those in strong iodine the smallest. These size-differences became accentuated during the rest of the experiment.

The development of hind-limbs was greatest in strong iodine in air, then followed weak iodine in air, then strong iodine in oxygen (differentiation, not size), then water in air, then weak iodine in oxygen, then water in oxygen. This order was preserved till 12.6.22 when the experiment as a whole was terminated. The following table gives some measurements. It will be seen that oxygen has affected both general growth and limb-growth, the latter more than the former. The gradational effect of increased iodine concentration is seen, especially as regards the fore-limbs and tail in air. No fore-limbs were produced even with strong iodine in oxygen.

It was noticeable that these older tadpoles were less affected by an atmosphere of oxygen than were the younger ones.

The experiment confirmed the previous result, viz, that an atmosphere of oxygen partially inhibits limb-differentiation, both that which occurs normally,

Table VI.

		Total length.	Body length.	Tail length per cent of total	Hind-limb length	Fore limbs and tail
B (strong iodine, O <sub>2</sub> ) (2 specs.)	av.	20.8	7.8	62.5	2.6	
	max.	22.5	8.0	—	2.8	
	min.	19.1	7.6	—	2.4	
C (weak iodine, O <sub>2</sub> ) (3 specs.)	av.	18.3	7.8	57.4	2.6	
	max.	24.0	9.1	—	2.5	
	min.	14.9	6.9	—	1.8	
D (water, O <sub>2</sub> ) (2 specs.)	av.	22.5	8.7	61.4	2.5	
	max.	24.0	9.3	—	3.0	
	min.	21.0	8.1	—	2.0	
E (strong iodine, air) (9 specs.)	av.	14.1	7.0	50.3	3.5	3 with both fore limbs & 8 with tails affected and 2 other specs died in advanced stage (short tail) but had decayed when found
	max.	20.7	8.3	—	4.2	
	min.	10.2	5.5	—	1.9	
G (weak iodine, air) (7 specs.)	av.	19.5	7.7	60.5	4.0	1 with both, 2 with left fore-limbs, 4 with tails affected
	max.	27.5	10.3	—	6.8	
	min.	10.2	5.3	—	2.3	
H (water, air) (10 specs.)	av.	24.5	9.4	61.6	3.5	
	max.	27.5	10.3	—	4.2	
	min.	21.6	8.1	—	2.9	

and that which occurs precociously as a result of iodine or thyroid treatment.

Four specimens were retained in strong iodine in oxygen and four in water in oxygen, to see whether complete metamorphosis would occur. Three in iodine and one in water died before 17.6.22. Metamorphosis of the one (unusually large) specimen in water occurred on 27.6.22. The others died before metamorphosis, the last on 30.6.22. Thus metamorphosis is *possible*, even to untreated specimens, in an atmosphere of oxygen.

*Expt. 8.*—On 13.6.22 an experiment was started to see what effect a moderate increase in the partial pressure of oxygen would exert. An arrangement was made whereby dishes could be subjected to a mixture of air and oxygen containing 40 per cent. of oxygen. Six dishes were prepared, A to C in 40 per cent. oxygen, D to F in air. A and D contained large, B and E medium, and C and F small tadpoles. They were fed for 24 hours only on thyroid and then placed in position with a little weed on 14.6.22. Ten days later, all were showing metamorphic changes, the large ones most, the small ones least, as before.

Very little if any difference could be detected between those in oxygen and those in air, *i.e.*, a partial pressure of 40 per cent. oxygen is not deleterious to the growth or metamorphosis of tadpoles, as is pure oxygen.

In order to get an approximation of the size of the animals used, seven tadpoles of each size used in the experiment were killed and measured on 13.6.22. The sizes thus arrived at were as follows :—

Table VII

		Total length	Body length	Length per cent of total	Hind-limb length
Large	av	22.3	9.1	59.2	0.6
	max.	25.0	10.0	—	1.3
	min	21.0	8.5	—	0.3
Medium	av	18.8	8.0	57.5	c 0.25
	max	21.5	8.8	—	0.5
	min	16.5	7.6	—	Just visible with eye.
Small	av	15.3	6.1	60.1	
	max	16.1	7.0	—	Just visible with lens
	min	13.9	5.1	—	

*Expt. 9*—As cyanides are known to depress oxidation, it was decided to test the effect of KCN in dilute solution upon the accelerated metamorphosis produced in iodine and thyroid.

Preliminary experiments were undertaken to find the lethal concentration. It would appear that the susceptibility of the animals to KCN distinctly increases with age. This may possibly have some relation to the fact that younger tadpoles are *more* susceptible to exposure to pure oxygen (p. 130). However, other factors such as temperature may influence the result, and the experiments must be repeated with large numbers before definite conclusions can be drawn.

As a result, N/80,000 was at first taken as the highest concentration of KCN for the metamorphic experiments. Later, however, it was found that too many deaths occurred in this, and first N/100,000 and finally N/140,000 was adopted. In all KCN experiments the fluid was renewed every day, and the containers covered with well-fitting covers.

*Expt. 10.*—The first thyroid and KCN experiment was started on 12.5.22. The mortality, however, was so high that the experiment was abandoned on 18.5.22. Another with lower concentrations of KCN was started on 17.5.22. Four vessels, with 10 tadpoles and 200 c.c. of fluid in each, were prepared as

follows:—A, tapwater. B, N/200,000 KCN. C, N/140,000 KCN. D, N/100,000 KCN. All animals were fed on thyroid. Unfortunately all the survivors in all the vessels were found dead on 25.5.22. The hind-limbs had only differentiated slightly in this time, but significant differences were observed. The following table gives measurements.

Table VIII.

		Total length	Body length	Tail length as per cent of total	Hind-limb length	Fore limbs and tail
A (tapwater and thyroid)	av	13.0	5.5	57.6	0.8	2, and 3 trace.
	max	14.3	6.0	—	1.3	All tails thin.
	(10 specs. surv.) min.	11.5	4.7	—	0.5	
B (N/200,000 KCN and thyroid)	av	11.3	4.6	59.2	about 0.6	1 and 3 trace.
	max	13.0	5.2	—	1.0	Most tails thin.
	(8 specs.) min.	8.2	4.3	—	about 0.4	
C (N/140,000 KCN and thyroid)	av.	12.0	5.0	58.4	< 0.5	1, 7, 1 trace.
	max.	14.0	5.3	—	about 0.7	Some tails thin.
	(6 specs.) min.	10.0	4.7	—	about 0.3	
D (N/100,000 KCN and thyroid)	av	11.8	4.9	58.5	about 0.25	0 forelimbs. Tails scarcely affected.
	max.	12.8	5.2	—	0.5	
	(5 specs.) min.	10.2	4.5	—	Trace	

Thus all the KCN solutions, even in eight days, seem to have reduced general growth, while, however, reducing limb-growth considerably more. The gradation in number of visible fore-limbs is well-marked.

*Expt 11.*—The experiment was repeated with slight variations, the chief being the substitution of iodine for thyroid treatment. It was started on 1.6.22, and ended on 12.6.22.

The following table gives the essential results. All vessels contained 200 c.c. of fluid. 10 tadpoles were placed in each at the outset. (Table IX.)

It is clear (1) that iodine inhibits tail-growth. (2) That it accelerates limb-growth. This is especially clear as regards the fore-limbs, but the hind-limbs of A and B were not only *relatively* larger than those of E and F, but were more highly differentiated. (3) That KCN alone inhibits general growth. (4) That it retards the limb-differentiation produced by iodine. It appears from F that extremely dilute solutions of KCN might actually have a favourable effect upon general growth, especially upon that of the tail (subtract body-length from total length). This, however, must be tested by future work.

Table IX.

		Total length	Body length	Tail length as per cent. of total.	Hind-limb length	Fore-limbs.
A Tapwater and 20 drops iodine solution (7 specs)	av	16.7	8.2	50.9	3.3	5
	max	20.0	8.6	-	4.1	
	min	15.5	7.3	-	3.0	
B $n/500,000$ KCN and 20 drops iodine solution (9 specs.)	av	14.9	8.3	44.3	3.4	2 less advanced than A
	max	19.0	9.2	-	4.2	
	min	12.0	7.3	-	2.0	
C $n/250,000$ KCN and 20 drops iodine solution (9 specs)	av	15.4	8.1	47.4	2.9	0
	max	20.5	9.1	-	4.2	
	min	12.2	7.5	-	2.0	
D $n/120,000$ KCN and 20 drops iodine solution (8 specs)	av	16.2	7.4	53.3	2.1	0
	max	20.2	8.8	-	2.9	
	min	13.3	6.0	-	1.1	
E Tapwater (10 specs)	av	20.1	9.1	54.7	3.3	0
	max	22.0	11.8	-	4.1	
	min	18.0	8.0	-	2.5	
F $n/1,000,000$ KCN (10 specs)	av	22.3	9.0	59.6	3.3	0
	max	24.5	9.5	-	4.3	
	min	20.5	7.8	-	2.5	
G $n/500,000$ KCN (5 specs)	av.	16.1	7.3	53.4	2.5	0
	max.	18.9	9.1	-	4.1	
	min	13.0	6.4	-	1.3	
H $n/120,000$ KCN (3 specs)	av	14.3	7.1	50.4	2.3	0
	max	17.1	8.2	-	3.7	
	min	10.5	6.1	-	1.2	

*Expt. 12.*—As the results of the experiment, owing to the variability of the animals, were not as clear-cut as could be wished, especially as regards the limb-growth, it was repeated as follows:—

Four dishes were prepared; all contained 200 c.c. fluid, with 16 drops of iodine solution; three were made up with various dilute solutions of KCN in tapwater, one with tapwater only as control. The solutions were changed daily. Ten tadpoles were placed in each dish. The hind-limb buds were just visible with a lens in a fair proportion of them. Food was provided. The dishes were kept at room-temperature.

The experiment was started on 13.6.22. On 24.6.22 the iodine dose was increased to 20 drops, on 28.6.22 again to 24 drops. This proved fatal; however, the differences between the various lots were by now well-marked, as is shown in Table X. Those in the strongest solution of KCN had been markedly

retarded in general growth : their limbs, however, had not developed at all, in spite of the iodine dosage. The growth of those in the  $n/250,000$  solution had been distinctly retarded. their limbs had grown, but very slightly. In the  $n/1,000,000$  solution general growth had been scarcely if at all affected. the length of the limbs, however, was only just over half that of the controls. It appears, then, that the acceleration of metamorphosis caused by iodine or to be precise, that part of it at least which is concerned with limb-growth - is

Table X. All vessels with the same strength of iodine in tapwater

		Total length	Body length	Tail length as percentage of total	Hind limb length
A Control (6 specs.)	av	17.0	7.1	58.2	1.6
	max	20.6	8.1	—	2.2
	min	13.0	6.0	—	0.4
B KCN $n/1,000,000$ (5 specs.)	av	16.8	6.9	58.8	0.9
	max	20.0	8.0	—	1.8
	min	14.0	5.8	—	0.3
C KCN $n/250,000$ (5 specs.)	av	16.2	6.5	59.8	0.2 0.3
	max	16.9	7.0	—	0.5
	min	15.3	6.0	—	trace trace
D KCN $n/140,000$ (5 specs.)	av	14.8	6.5	56.2	( 1 0 1)
	max	15.8	7.0	—	trace —
	min	13.0	5.2	—	trace —

markedly interfered with by cyanide depression. The growth of the body is also interfered with, but to a relatively much smaller extent. even very dilute solutions of KCN reducing limb-growth by nearly 50 per cent, while scarcely or not at all affecting the general body-size, and stronger solutions of KCN almost entirely inhibited hind-limb growth.

*Expt. 13.* -An experiment was also undertaken to see the effect of alcohol upon thyroid-accelerated metamorphosis. It was first determined that a 2 per cent. solution of absolute alcohol in tapwater was lethal to medium-sized tadpoles within three days, while 3 per cent. alcohol killed within 12 hours. Four vessels were prepared as follows (200 c.c. in each). A, tapwater and meat. B, tapwater and fresh ox-thyroid. C,  $\frac{1}{2}$  per cent. alcohol in tapwater and fresh ox-thyroid. D, 1 per cent. alcohol and fresh ox-thyroid. 10 tadpoles were placed in each dish (23.5.22). Death ensued in the alcohol solutions after 11 days. The animals were not measured, but inspection revealed marked differences. In A, no metamorphic changes had occurred. In B, meta-



metamorphosis was nearing its critical point (hind-limbs well developed, tail thin). In C, hind-limbs with digits had appeared. In D, the tails were longer than in C or B, the bodies less fiddle-shaped, and only traces of limb-buds were to be seen. Thus alcohol, like KCN, markedly retards thyroid-induced metamorphosis.

*Expt. 14.*—Since the ox-thyroid used appeared to decay more rapidly in alcohol than in water, the following variation of the last experiment was carried out. Three vessels were prepared as follows, with 200 c.c. and 10 tadpoles in each. In A and B the animals were fed on thyroid, in C on meat. The thyroid and meat feeding was continued, in plain water, from 3.6.22 to 6.6.22, when the diet was changed to boiled lettuce. The fluid in A was at the same time changed to 1 per cent. alcohol, in B to  $\frac{1}{2}$  per cent. alcohol.

The animals were preserved and measured six days later (12.6.22), with the following results:—

Table XI

		Total length	Body length	Tail as percentage of total.	Hind limb length	Fore-limbs
A (1 per cent. alcohol and thyroid)	av	16.0	8.0	50.0	2.0	L. fore-limb present in 6 out of 10 specs
	max	20.0	11.0		3.5	
	min	13.0	7.0		0.9	
B ( $\frac{1}{2}$ per cent. alcohol and thyroid)	av	13.9	7.5	46.1	2.3	No fore-limbs visible (10 specs)
	max	19.5	8.1		3.1	
	min	11.5	6.8		1.7	
C (Water and meat)	av	21.5	8.7	59.6	1.0	No fore-limbs (10 specs)
	max	23.0	9.5		1.2	
	min	20.0	7.9		0.9	

Thus the increased retardation of thyroid-metamorphosis with increased alcohol concentration is again evident.

*Expt. 15.*—Another experiment was also carried out to test the effect of alcohol on the acceleration of metamorphosis produced by iodine.

The result was in accordance with expectations based on the KCN experiments. It may be briefly summarised as follows:—

Five vessels, 200 c.c. tapwater or alcohol in tapwater; 16 drops iodine solution, and 20 small-medium tadpoles in each. Experiment started 9.5.22.

A.— $1\frac{1}{2}$  per cent. alcohol, all died before 2.6.22.

B.— $1\frac{1}{4}$  per cent. alcohol, all died before 6.6.22, unjointed limb-buds only.

C.—1 per cent alcohol, 3 alive 6.6.22, jointed hind-limbs, but no digits

D.— $\frac{1}{2}$  per cent alcohol, nine alive 6.6.22, hind-limbs with well-formed digits

E.—Tapwater, unfortunately died early owing to an accident

Thus here, too, the gradational effect of alcohol was clear.

#### 6. *Experiments on Triton Cristatus.*

As is well known, male *Triton cristatus* develop a fine serrated fin-crest all along the back, together with a large fin on both upper and lower margins of the tail-*ve.*, in the same position as the fin of larval Urodeles (although, as might be expected, of somewhat different shape and colour). It was decided to try the effect of thyroid treatment on this structure.

A number of newts (*T. cristatus*) were brought into the laboratory in the last week of April, 1923. Some were injected with physiological saline as controls, others with thyroid, a few were made to eat thyroid in addition. All were kept at room temperature (13° to 14° C.).

The animals were weighed at intervals, and the following measurements taken—total length, height of crest (back fin), total height of tail with fin

The results are presented in tabular form (Table XII)

The results are not wholly satisfactory, owing to the fact that *T. cristatus* kept in captivity lose weight and show regression of their crest and tail fin without treatment. However, it is clear that in total weight-loss and fin-regression within 32 days, the thyroid treatment produces no marked acceleration. The four thyroid-treated specimens averaged 37.1 per cent. weight loss, as against 29.5 per cent. for the three controls, but one of the controls lost more weight than one of the animals injected four times with thyroid, so that the difference is probably not significant. Very similar averages are given by the other measurements, and similar deductions may be drawn.

We may sum up by saying that there may exist a slight acceleration of weight-loss and fin-resorption as the result of thyroid treatment, but that the effect is possibly absent and certainly not more than slight.

There is nothing similar to the specific action of thyroid on the fin of larval Urodeles. It is of course known that the growth and maintenance of the fin in adult male Urodeles in the breeding-season is dependent upon the testis. Thus we have the remarkable phenomenon of an almost identical organ in quite identical position controlled by two different mechanisms in two phases of the life-cycle.

In the larval period it is not known to be dependent on any endocrine organ for its growth or maintenance, but is broken down by excess of thyroid while

Table XII.

No.	Treatment	Weight		Total length (mm.)		Tail height (mm.)		Crest height (mm.)	
		April 28	May 30	April 28	May 30	April 28	May 30	April 28	May 30
			Per- centage loss					Per- centage loss	
1	Injected NaCl once	7.8	5.8	114	114	15	10.7	6-	2.5-
2	Injected NaCl once	9.8	6.2	123	124	18	9-	51	2.5
3	Injected once, 0.5 grain thyroid	8.5	5.1	119	117	14	8.5	39	1.5+
4	Injected once, 0.3 grain thyroid	6.3	4.0	105	101	15	8+	45	2-
5	Injected four times, 2.75 grains thyroid in all, fed thyroid once	10.4	6.1	124	123	17	8	53	2
6	Injected four times, 1.25 grains thyroid in all	6.9	4.8	112	111	16--	8.5	46	1.5
7	Not injected (brought into laboratory March 3): then weighed 12.6 grains, tail height 14 mm., crest height 7.0 mm.	10.8	9.3	140	140	13.5	12	14	2-
			(from Mar 3)					(from Mar 3)	(from Mar 3)

in the adult phase it is dependent upon the testis both for growth and maintenance, and is not broken down by excess of thyroid

## 7 Discussion

I.—Failure of iodine to effect the metamorphosis of Axolotls. The strains of Axolotls used were all of the Old Mexican sub-species, which does not metamorphose in response to slight environmental changes, as is the case with the New Mexican sub-species used by Swingle (1923). High temperature, changes of temperature, stimuli such as that of travel, inanition—none of these, singly or in combination, caused metamorphosis, although high temperature ( $30^{\circ}$  to  $31^{\circ}$  C) almost always induced loss of weight and sometimes slight pre-metamorphic symptoms

In Anura, either immersion in inorganic iodine solutions or the ingestion of iodine crystals will cause acceleration of metamorphosis, which is very marked if the concentration is high. This is even true for thyroidectomised frog tadpoles (Swingle, 1918, 1919), although here, as might be expected, there is a threshold concentration for metamorphosis, and the acceleration with the same effective concentration is less than in unoperated animals.

The failure of the Axolotl to metamorphose under similar treatment is thus an important point of difference. The thyroid of Axolotls are quite large, and contain abundant colloid. In the New Mexican race, at least, they are physiologically active, for they will induce metamorphosis in tadpoles when engrafted (Swingle, 1923). Jensen (1920), however, asserts for the Old Mexican sub-species that after a certain period the thyroid degenerates and becomes physiologically inert. Further work is needed before the matter is cleared up.

In any case, the experiments here recorded make it quite clear that inorganic iodine, in the strongest concentration which the animal will tolerate, or given in large quantities with the food, will not induce metamorphosis. On the other hand, certain preliminary symptoms of metamorphosis are induced by the treatment. This implies that iodine operates in the same kind of way in the Axolotl as in frog tadpoles, but that its effect cannot reach the intensity necessary for complete metamorphosis.

Hirschler (1922) states that he has induced complete metamorphosis in Axolotls by introducing inorganic (elemental) iodine intraperitoneally. This experiment merits repetition. In any case, two interpretations are possible. Either the effective concentration of iodine that can be obtained in the body is greater by this method, or else Hirschler was using an unusually susceptible strain of Axolotls. I have myself found, using standardised thyroxin solution,

that different strains of the Old Mexican sub-species may differ considerably with regard to the minimal dose per unit weight needed for metamorphosis. If Hirschler had employed a particularly susceptible race, it might have reacted to injection by complete metamorphosis, while the strain which I used might have confined itself to the pre-metamorphic changes which I have described.

There are various possibilities to account for the failure of Axolotls to metamorphose under iodine treatment. Swingle believes that the thyroid in some unexplained way is inhibited from releasing its secretion into the blood-stream. This does not appear to be valid, or at least not to be the main cause, in the Old Mexican strain, since in these auto-transplantation of the thyroid, which according to Swingle (1923) always causes liberation of at least most of the contained secretion, does not produce metamorphosis (Hogben, 1922). The possibility that the thyroid of the Axolotl does not utilise iodine for the production of its secretion in the same way as that of Anura is very improbable, and lacks any facts in its support.

There remain two other possibilities. Either it is impossible for the thyroid of the Axolotl to attain to the same size relative to the rest of the body as the thyroid of Anura, or else the tissues which respond to the metamorphic action of thyroid (*e.g.*, fin, gills, gut by regression, skeleton, brain, eyes by progressive changes) demand a higher threshold of thyroid concentration in the Axolotl than in Anura.

It appears to me that in the present state of our knowledge it is impossible to decide between these alternatives. Analogies can be found for both. It is a commonplace of developmental physiology that an organ may reach very different relative sizes in two related species (chelæ of different species of crabs; toes of different primates and human races), or in the two sexes of the same species (chelæ of male and female crab, mammalian clitoris and penis), or in individuals of the same species and sex (antlers of stags of the same age, wisdom teeth of man). It may perfectly well be that the thyroid of the Axolotl, even though supplied with unlimited iodine (the probable limiting factor in thyroid development), may be unable to develop to a relative size large enough to induce metamorphosis, whereas the relative growth-rate of the frog tadpole's thyroid is much greater, and it does not reach equilibrium so soon (compare the difference between the different relative growth-rates of male and female abdomen and chelæ in crabs—Huxley, 1924, *a* and *b'*).

On the other hand, it is quite clear that homologous tissues in related species may differ entirely in their reaction to one and the same substance. The most familiar example is perhaps the difference in the behaviour of

the limbs in Anura and Urodela as regards thyroid secretion. In Anura the growth rate of the limbs in the absence of the animal's thyroid is, though not *nil*, yet very low—a good deal lower than that of the organism as a whole (Hoskins and Hoskins, 1919). Above this, their rapidity of growth is proportional to the concentration of thyroid to which they are exposed, up to quite a high concentration (in the normal animal their relative growth-rate is somewhat faster than that of the rest of the body after the thyroid has become functional, still faster when the development of the thyroid is accelerated by iodine, or when extraneous thyroid is added) In both these latter cases the proportionality of acceleration to concentration has been shown (Swingle, 1919; Huxley, this paper; Romeis, 1913 to 1915) In Urodela on the other hand, the growth-rate of the limbs appears always to be close to that of the body as a whole, and is not at all or only negligibly influenced by removal or addition of thyroid

An even more startling example is that afforded by the Perennibranchiates. Neither *Necturus* (Huxley and Hogben, 1922; Jensen, 1916) nor *Proteus* (Jensen, 1916) can be metamorphosed by the strongest doses of thyroid. The fins and gills do not react to thyroid in the way in which they do in all other known Amphibia. Thus there is again nothing impossible in the supposition that the method by which the neoteny of the Axolotl is produced has consisted in a lowering of the sensitivity of the tissues involved in metamorphosis to thyroid. Of course, a combination of the last two methods might equally well prove true. The problem can only be resolved by making accurate comparative studies as to the minimal dose of standard thyroid preparations needed to effect metamorphosis in intact and thyroidless animals of various species in both groups of Amphibia at various periods of their growth.

There is finally the fact discovered by Uhlenhuth (1921) that in Urodela the thyroid is in an almost completely passive condition throughout larval life, but suddenly passes, just before metamorphosis, into great activity. The cause of this activation has not yet been found. I have had the opportunity, through Dr. Uhlenhuth's kindness, of seeing preparations and other data as yet unpublished, and agree that they confirm his interpretation. In the Anura, on the other hand, all the evidence points definitely to a considerable activity of the thyroid throughout larval life (after the internal gill stage), followed possibly by a certain additional activity before and during metamorphosis.

It is highly probable that the difference in the effect of iodine on the larvæ of the two groups is partly correlated with this difference in the behaviour of

their thyroids; but the other factors above mentioned must also be reckoned with.

A point worth emphasising is the differential effect of the depressants used (KCN and alcohol) upon the growth of the limb-buds, as compared with the body and tail. It is to be noted in the first place (*e g.* Tables VIII and XI) that KCN inhibits all metamorphic processes, the destructive tail-resorption as well as the constructive limb-growth, so that the tail-reduction must be dependent on the increased metabolism induced by thyroid, and not a dedifferentiation similar to that which can be obtained in sea-urchin larvæ, etc (Huxley, 1922) by KCN and other depressants. However, the differences in the growth of the limb-buds obtained by depressants are far greater than those produced in other organs. This is doubtless correlated with the fact that any active constructive process, such as rapid cell multiplication, would be directly affected by KCN, and that such active cells are relatively more susceptible than those of the general body (Child, 1915).

It should also be noted that during the length of time occupied in these experiments, percentual (relative) tail-length is hardly if at all affected by iodine treatment, and only in some cases with thyroid. This implies that a higher threshold concentration of thyroid hormone is necessary to affect the tail than to affect the limbs. This in a sense is obvious in normal metamorphosis, where (particularly in such large forms as bull-frogs) the tail remains intact for weeks, even after all four limbs are visible and large. None the less it is an important fact, and has important theoretical bearings.

The effect of iodine treatment is presumably to permit a greater relative increase in the growth of the animal's own thyroid, leading to a gradual greater liberation of thyroid hormone, iodine being thus normally the limiting factor in thyroid growth; while the effect of thyroid treatment is to raise the thyroid concentration suddenly by a definite amount.

The growth of the Anuran thyroid during the larval period is of considerable general interest, since we have here an organ with the following properties: (1) It starts to be active only from a certain definite period of development; (2) after this its relative growth-rate is larger than that of the body as a whole, up to the critical phase of metamorphosis; (3) however, iodine acts as a limiting factor to this growth-rate; (4) different organs respond to different levels of concentration of its active principle, the limb buds starting at once, the tail not responding until a relatively high concentration is reached.

Somewhat similar effects are noted with regard to other ductless glands. It has, for instance, often been found puzzling that the gonads should exert

certain effects only at puberty, while other effects (*e g.* on the accessory reproductive organs) have been exerted by the same gland long previously (see Crew, 1923, for discussion of early effects) It is, however, known (Hatai, 1913) that the gonads, in the rat, undergo a period of very rapid growth relative to the rest of the body, from soon after birth to just before puberty, and it is quite possible that the response of the accessory organs is like that of the limbs in the tadpole, while the secondary sexual characters only respond to greater concentrations. In any event, the facts in Anura must be borne in mind in any discussion of developmental physiology in relation to endocrines.

### 8 Summary.

1. *Effects of air-breathing on the Axolotl.* (a) Sixteen Axolotls were forced to breathe air over long periods; none, however, metamorphosed as they did in the experiments of v. Chauvin and Boulenger. It is suggested that genetic differences exist among different strains of Axolotl in regard to readiness to metamorphose, and that these are responsible for the discrepant results. The fact of numerous failures among the results of the authors who obtained some successful results supports this interpretation.

(b) Interesting morphogenetic effects of the terrestrial environment upon the aquatic tissues were observed. The most striking of these is the falling over of the fin to one side, and its subsequent *complete fusion* with the skin of the back. Evidence of this fusion can be detected in sections long after no trace is visible externally. Fin-disappearance is thus effected in quite different ways in normal metamorphosis and as a result of enforced air-breathing.

2. *Metamorphosis under narcosis.* -By using urethane it was found possible to keep frog tadpoles under complete narcosis for over a week (up to 10 days). If such animals were previously treated with thyroid, metamorphosis proceeded at a normal rate in spite of the narcosis. This is also in spite of the fact that the death-rate of thyroid-treated tadpoles is markedly increased by urethane narcosis over long periods.

By means of the Barcroft differential gas apparatus, the oxygen consumption of the animals was measured. It was found that exposure for 24 hours to moderately strong thyroid solutions four to six days previously increased the oxygen consumption of half-grown tadpoles by about 100 per cent. per unit of body weight (about 50 per cent. per individual, owing to the fact that great weight-loss occurs); and that complete urethane narcosis depresses the oxygen consumption of both normal and thyroid-treated tadpoles by about 40 per cent. These results are preliminary only.



3. *Effect of Iodine and Oxygen upon Axolotl metamorphosis*.—Exposure of Axolotls to the strongest tolerated solutions of inorganic iodine will not produce metamorphosis, neither will the giving of large quantities of iodine crystals with the food. This is still true when the experiments are carried on in an atmosphere of oxygen.

On the other hand, changes which may be called pseudo-metamorphic do occur under these conditions. They consist in reduction of fin and gills, protrusion of eyeballs, and other of the changes associated with early stages of normal metamorphosis. However, the critical stage is never reached, and the irreversible moult does not occur. These pseudo-metamorphic changes are greatest in the stronger iodine solutions, and are increased in an atmosphere of oxygen.

4. *Experiments with iodine and thyroid in conjunction with other agents in tadpoles*.—Swingle and Romeis had shown that the metamorphic effects of iodine and of thyroid upon Anuran tadpoles were quantitative. These results are confirmed. The quantitative effect of various agents was then tried out upon the rate of metamorphosis induced by definite strengths of iodine and of thyroid in frog tadpoles.

Quantitative estimations of rate of metamorphic change were made by measuring the length of body, tail, and limbs at definite intervals.

(a) *Oxygen*.—Exposure to an atmosphere of oxygen is in itself deleterious to tadpoles, and causes poor growth and emaciation: this is more marked in earlier stages. Pure oxygen retards the metamorphic changes induced by iodine and thyroid, although metamorphosis is possible to tadpoles in this environment. In a mixture of air and oxygen containing 40 per cent. oxygen, metamorphic rate was normal.

(b) *Potassium cyanide*.—Susceptibility to this appears to increase with age. It inhibits general growth. When given in conjunction with iodine or thyroid it retards metamorphic changes very materially, relatively much more than it does general growth.

(c) *Alcohol*.—Like KCN, this quantitatively retards the precocious metamorphosis produced by iodine or thyroid, in proportion to the concentration used.

5. *Experiments with Triton Cristatus*.—Thyroid administration to adult males during the breeding season produced no significant acceleration of the fin-regression and weight-loss which always occur in this species in captivity—i.e., the regression of the fin in adult *T. cristatus* is not determined by the same causes as in the metamorphosing larva of the same species.

6. *Discussion*.—The different reaction of different tissues in various species to thyroid and other agencies is discussed, and it is pointed out that tissue speci-

city is of equal value in determining a particular reaction with the activity of a particular endocrine gland—*e.g.*, in Anura the limbs are affected by a low, the tail only by a high concentration of thyroid. The Urodele larval limbs are not affected at all. The same organ may respond to different endocrine secretions at different periods—*e.g.*, the fin of *Triton*.

In conclusion, I wish to express my indebtedness to the Royal Society for a grant, and to my assistant, Mr. Kempson, for his care of some of the animals.

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## EXPLANATION OF PLATES.

<i>bl.v.</i> = blood vessel.	<i>m.g.</i> = mucous gland.
<i>c.t.</i> = connective tissue.	<i>msc.</i> = muscle.
<i>ep.</i> = epidermis.	<i>sp.</i> = spaces round axis of bent fin.
<i>f.a.</i> = fin-axis.	

## PLATE 14.

All figures are microphotographs of sections through the back fins of Axolotls after exposure to air for some weeks. All show epidermis, mucous glands, mesodermal fin-axis, and muscular tissue.

- FIG. 1—Two sections, close together, through the same fin—A, medium power; B, high power. In A the tip is still free. Note concentric arrangement of epidermal cells round bent tip of axis; blood-cells in bent axis-tip.
- FIG. 2.—Fin of another animal. Complete fusion, but original end of fin marked by sudden thinning of epidermis. Tip of axis bent over, with concentric arrangement of epidermal cells near it. Low power.

## PLATE 15.

- FIG. 3.—The same fin, cut nearer the head: two sections, close together—A, low power; B, medium power. The original tip of the fin is marked as in (2), but no trace of bent-over condition remains in the tip of the axis, or of concentric arrangement of epidermal cells near it.
- FIG. 4.—Section (low power) through the fin of another specimen at the junction of tail and body. The dorsal side is towards the left. The fin had fallen over to the R. on the back, to the L. in the tail. Note spaces produced within the fin by the opposing tensions.



FIG. 1A.

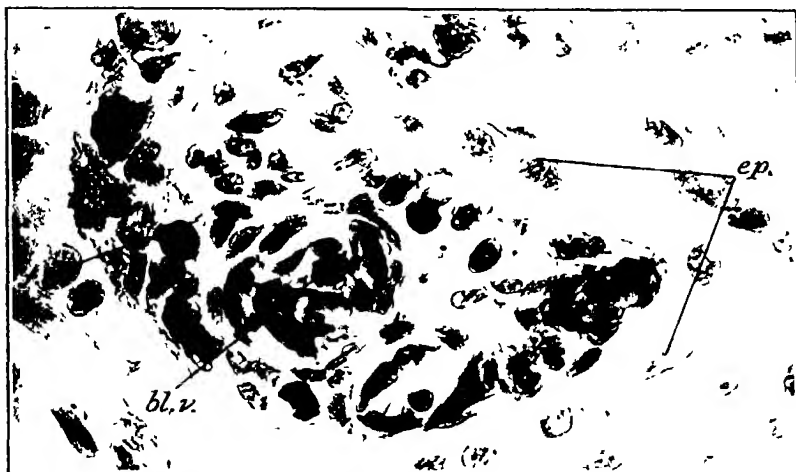


FIG. 1B



FIG. 2



FIG 3A.

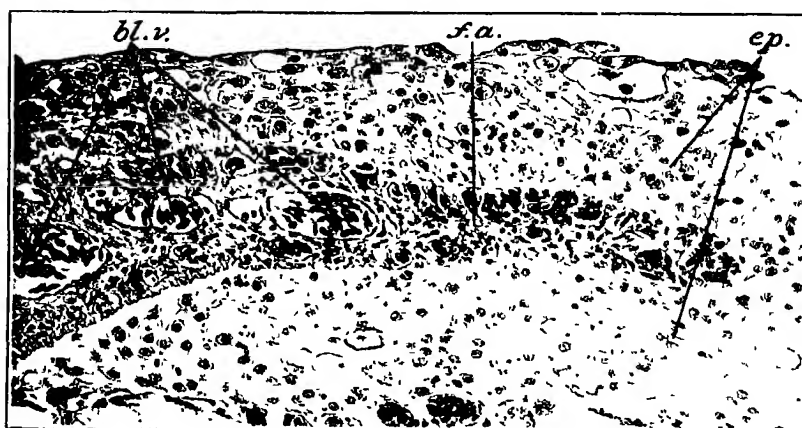
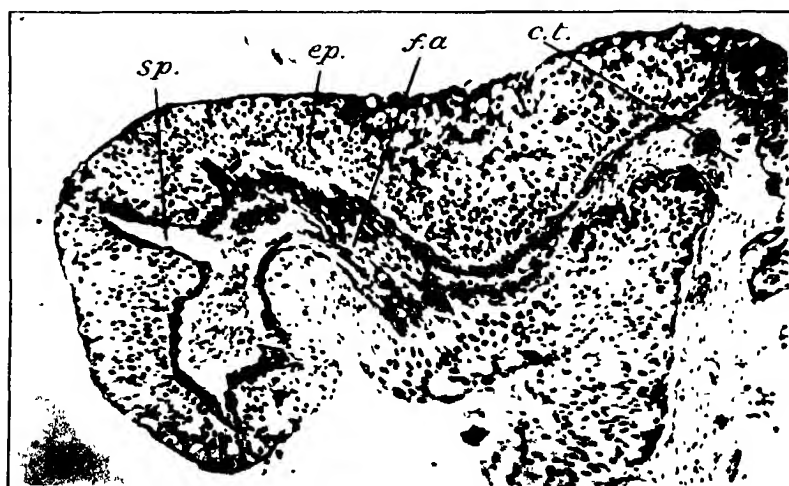


FIG 3B.



*dorsal*

FIG 4.

*The Effect of Vitamin B Deficiency on Reproduction.*

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(PLATES 16 AND 17.)

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(1) *Introductory.*

A number of investigators have observed atrophy and degenerative changes in the testes of animals fed on diets deficient in the factor termed Vitamin B. Funk and Douglas (5) reported that the testicles of pigeons showing symptoms of avian polyneuritis were greatly atrophied, and that the tubules were diminished in size and contained no spermatozoa. Drummond (3) observed a similar condition in the testes of adult rats deprived of vitamin B, whilst McCarrison (8) found the atrophy of the male gonads to be very much more severe in pigeons with beri-beri than in monkeys with that disease.

Allen examined the testes of rats which had been fed on Osborne and Mendels' diet deficient in vitamin B, and found considerable derangements, to which further reference will be made in the section on Histology.

Microscopic examination of the tissues of animals which have been deprived of an adequate supply of vitamin B reveals the fact that the degenerative changes in the testicles are in most cases the pronounced abnormality. In this connection it should be noted that in all cases of animals actually showing beri-beri symptoms, we have observed at post-mortem examinations that the congestion and varicosity of the venous system was far more pronounced in the case of the spermatic vessels than elsewhere. Confirmation of the degenera-

tive changes in the testicles of pigeons has been presented by Novaro (9) and by Portier (12), the latter having shown that if substances containing vitamin B are added to the diet before the abnormal conditions become too acute, recovery of the testes to an almost normal condition may occur.

The sterility that would be expected to follow severe degenerative changes in the male gonad has actually been observed by Drummond (3) and by Allen (1). The former reported that male rats deprived of vitamin B for more than 14 days cease to breed with females normally fed. His actual results were :—

No. of Male	Days upon inadequate dietary before given access to females	Results of Mating
1	0	2 pregnancies, 15 young.
2	7	1 pregnancy, 3 young
3	14	No pregnancy.
4	21	"
5	28	"
6	35	"

As regards this original experiment, however, it should be noted that the male was mated with two females for 48 hours only, and as the female rat has an œstrous periodicity of about five days, this can scarcely be regarded as a fair test of the fertility of the male. Nevertheless, in the light of more elaborate experiments, it is safe to say that the results pointed in the right direction, and a suggestive correlation with Allen's histological observations was obtained.

There, however, the question of the effects of vitamin B deficiency on reproduction was allowed to rest. Recently the present writers were led to investigate the subject in some detail, and the present paper is the outcome of the experiments carried out with this object.

The points dealt with include—

- (1) Histology
- (2) Fecundity.
- (3) Fertility.
- (4) Sex-ratio.
- (5) Weight of young.

Owing to the fact that males on a diet totally deficient in vitamin B live for a short time only, and will breed for even less time, it was decided to put animals on a series of diets graduated between complete sufficiency and complete

deficiency, so that data could be obtained over a greater period, though naturally for less severe conditions. These graduated diets are further discussed in the next section.

One further point remains to be mentioned, *i.e.*, the effects of vitamin B insufficiency upon reproduction in the female. Drummond (3) has shown that pregnant does placed upon deficiency diets a few days before parturition suffer considerable derangement of the normal breeding function, and that the young are also affected. It appears to be impossible to breed from a female which has been on the diet any length of time, and the recent researches of Evans and Bishop (4) suggest an additional reason for this. It is as certain as an *a priori* statement can be that, even if a doe on a B deficient diet became pregnant, the death and re-absorption or abortion of the embryos would occur, owing to the malnutrition of the mother, and that tertiary sterility would be set up.\*

The study of the effect of deficiency of vitamin B on the reproductive capacity of the female is, however, complicated by the fact, observed by Evans and Bishop (4), that when fed on an artificially constituted ration satisfactory for normal growth, rats may fail to reproduce satisfactorily. When pregnancy is established the embryos are usually reabsorbed before full term. The striking researches of Evans and his colleague have made it apparent that for normal reproduction in this species, a dietary factor which can be sharply differentiated from the three or four recognised vitamins is requisite. The new factor has been provisionally termed vitamin X † It is obvious, therefore, that until some source of vitamin X is available, which does not also contain other recognised dietary factors, it will be well-nigh impossible to study the influence of a deficiency of vitamin B on reproduction in the female. Some of the earlier experiments of Evans and Bishop clearly illustrate this difficulty. Since, however, the male rat would appear to be independent of vitamin X for effecting reproduction, the experiments to be described may be regarded as

\* Considered in the broadest manner complete sterility of a pair of animals may be due to any of three causes:—

- (a) Inability of adults to produce gametes,
- (b) Inability of adults to effect fertilization,
- (c) Inability of female to perform gestation,

and for convenience these three types of sterility may be known as (a) primary, (b) secondary, (c) tertiary (Parkes 11).

† Confirmation of the essential facts reported by Evans and Bishop has been obtained by us (see further, Section (8), Discussion). It would appear desirable to label his newly discovered dietary factor Vitamin E, and so maintain the regular sequence of lettering.



free from this complication since they concern artificially nourished males mated to females fed on a normal diet.

## (2) *Methods and Material.*

The composition of the artificial dietary employed for feeding the male rat was one that has been used with success in this laboratory for a number of years.

The composition is as follows:—

Purified caseinogen	20 parts
Rice starch	55 „
Butter fat	15 „
Salt mixture*	5 „
Lemon juice	5 „

The only constituent of this ration which is likely to contain vitamin B is the caseinogen, and especial care was taken to purify this substance by repeated extraction with alcohol. The deficiency of this diet as a source of vitamin B is at once apparent from the failure of animals to thrive when fed upon it, and by the prompt restoration of normal growth and nutrition when an adequate supply of the missing vitamin is added.

In order to study the effect of a graded deficiency of vitamin B a series of rations were compounded containing different proportions of a commercial yeast extract (marmite), which has been found to be a convenient form in which to administer this particular accessory factor. Long experience has taught us that male rats will show normal growth, nutrition and reproductive capacity when fed on the diet described above supplemented with 4–5 parts of yeast extract.

All female rats employed in these breeding experiments were fed on a satisfactory mixture of natural foodstuffs.

In some earlier trials we employed females fed on what had been regarded as

\* The composition of the mineral salt mixture is:—

	Grams
Sodium chloride	5.2
Magnesium sulphate	8.0
Sodium dihydrogen phosphate	10.4
Dipotassium hydrogen phosphate	28.6
Calcium phosphate	16.2
Calcium lactate	39.0
Ferric citrate	3.5
Manganese sulphate	trace
Potassium iodide	trace

a complete artificial ration (B + 5), but they failed to show normal fertility. The explanation is undoubtedly given by the researches of Evans and Bishop who have pointed out that such a diet is deficient in a factor necessary for fertility in the female.

The experiment was started with twenty buck rats which were divided into five groups of four each. These groups were put on five grades of deficiency, *i.e.*,

(a)	Basal diet	No vitamin B	(B )
(b)	„ „	+ 1 per cent yeast extract	(B + 1)
(c)	„ „	+ 2 „ „	(B + 2)
(d)	„ „	+ 3 „ „	(B + 3)
(e)	„ „	+ 4 „ „	(B + 4)

5 per cent. marmite is considered to supply vitamin B in an abundance. In 15 days one rat from each group had a unilateral castration performed on it so that histological observations could be made on the beginnings of degeneration in the testes. At the end of four weeks the remaining testes of the same five rats were removed and the animals destroyed. By this method of partial castration, progressive histological disturbance in the testes could be studied with the least probability of extraneous variations appearing to confuse the issue. At later dates successive batches of fresh bucks were put on to the deficiency diets. Since it soon became obvious that the extreme categories of the first series would give few results of interest, a second series of animals was put on the following range of diets :—

(a)	Basal diet	+ 0·5 per cent. yeast extract.
(b)	„ „	+ 1·0 „ „ „
(c)	„ „	+ 2·0 „ „ „

Subsequently a group were put on basal diet + 3 per cent. yeast extract. Finally, in an attempt to study more accurately the results of total or nearly total deficiencies, further groups of animals were fed on basal diet alone, and basal + 0·5 per cent. yeast extract.

During the whole time that these bucks were on the deficient diet a check was kept on their condition by regular weighings, and in each case it was endeavoured to mate the animal before any really severe symptoms of malnutrition set in. In the case of the rats on the diet free from vitamin B, this meant mating very soon, since none of these animals survived more than six weeks, and no litter was conceived from these animals after one month of

dieting. Of the rats on basal diet + 0.5 per cent. yeast extract the survival was nine weeks, and the limit to the period of fertility five weeks. On basal + 1 per cent. yeast, life is obviously possible for a considerable time. One rat on this diet was still surviving after 23 weeks, and was fertile, in spite of the early removal of one testis, after 20 weeks. It was then put on to normal diet and recovered, after a sample of the remaining testis had been taken. On basal diet + 2 per cent. and upwards of yeast, it is apparently possible to maintain life for a long time, and animals on these diets were not mated until they had been dieted for a month or six weeks. The weight curves of the dieted animals followed courses which have been well established by previous work, and further details of this aspect of the subject are not given here.

Animals on diets sufficient for life were finally put back on to normal food and allowed to recover, samples of the male reproductive organs being taken for each degree of deficiency at

- (a) The end of dieting.
- (b) The early stage of recovery.
- (c) The final stages of recovery.

In the case of one animal, P 5, which was on a basal + 1 per cent. yeast extract diet, three samples of testis were taken in all. After two weeks of dieting the right testis was removed for examination, the operation being followed by good recovery. At the twenty-third week of dieting, by which time it had produced six litters, a sample was taken from the remaining testis by the following method:—The tunica albuginea was punctured with a scalpel and a small part of the mass of tubules was squeezed out and kept for examination; the puncture was then cauterised and the testis replaced. Though the operation was followed by no fatal effects, recovery was not good, and the subsequent sterility of the animal was probably due to this manipulation rather than to any incomplete recovery from the deficiency diet. After six weeks on normal food, the animal was killed and the rest of the remaining testis preserved for examination.

When both sexes are kept on a diet deficient in vitamin B, no breeding takes place, owing to the fact that, even if fertilisation is effected, the embryos fail to develop to full time. In investigating, therefore, the action of B deficiency on the male reproductive powers it was essential that the females should be as normal as possible, and to this end, as an arbitrary limit, 130 gm. was taken as the minimum weight for mating does, and a certain amount of time for recuperation was allowed between successive litters.

Since the males and females were on different diets, some difficulty arose in the actual mating. At first it was hoped to be able to employ Long and Evan's (7) vaginal smear method to limit the stay of the doe with the buck to the actual period of œstrus, but the labour involved by this method was found to be greater than the advantages of its use. In practice, it was found sufficient to leave the doe with the male on the deficient diet and to administer vitamin B to the doe separately once a day in the form of yeast extract. The females did not take very readily to this procedure, but it was found possible to induce them to take enough yeast extract to make up the deficiency.

After being mated for one week the doe was separated from the buck and kept by herself until such time as pregnancy or non-pregnancy could be ascertained. Regular weighing was found to be sufficient for this purpose ; again a saving of labour as compared with the vaginal smear method, and watching for the "placental sign" of Long and Evans. Since the embryo is not implanted for some days and the mass of young conceptuses is small, it is, of course, impossible to detect the early stages of pregnancy by weighing, but in the vast majority of cases the method is accurate enough for the purposes of the present investigation. After parturition the following data were observed :—

- (1) Size of litter.
- (2) Sex of young.
- (3) Weight of young.
- (4) Still-births.

The doe was then allowed to rear the young until weaning time at four weeks, the weights of the young being taken at intervals for the purpose of examining their growth in relation to the normal. The subsequent history of these offspring from normal does by B deficient bucks was not followed up, because the question of permanent injury to the young was not under consideration.

### (3) *Histology.*

Allen (1) has described in some detail the degeneration which occurs in the testes of animals kept on a diet deficient in vitamin B, and hence it is not proposed here to enter into a further description of the cytological changes involved. It is intended to consider how far the various groups of rats on the various degrees of deficiency show the successive stages of degeneration as figured by Allen.

Post-mortem examination of any of the rats which had been on a severe deficiency revealed remarkable stagnation of the venous system, this being

specially noticeable in the spermatic vessels, and in the vessels of the testis tunica, which often had the appearance of being "bloodshot." Superficially the testes look normally plump, but actually, as noted by Allen, this is due to the fact that the tunica is distended by a serum-like fluid which escapes when the tunica is pierced. In reality, the testis tissue is shrunken.

It was noted by Allen, and made abundantly clear by our own material, that the degeneration in the tubules is far from regular. Tubules showing an extreme stage of degeneration may be found alongside tubules showing a fairly normal condition. The cause of this variation in a single testis is obscure, but it must be due to differences in the toleration of vitamin deficiency. It is all the more peculiar as no particular part of the testis seems to be more immune or less immune than the rest. A practical consequence of this variation in the same testis is that it is only roughly possible to compare one testis with another, and only roughly possible to trace the correlation of the histological condition of the testes with the degree of vitamin deficiency of the diet and with the breeding performance of the animal.

From the account given by Allen it would appear that the degeneration starts in the germ cells and afterwards spreads to the Sertoli tissue. At any rate, in the ultimate stage of degeneration only atrophic Sertoli nuclei are found. In an intermediate stage of degeneration the Sertoli tissue is very abundant, and sometimes completely blocks the tubule by forming a core in the lumen. Later the core disappears entirely, leaving the Sertoli nuclei very much reduced in size and number and practically confined to the sides of the tubule. Since the animals die by the time this stage is reached, this appears to be histologically the final stage of degeneration.

On the basis of this preliminary discussion the material presented by the rats used in the present experiments may be considered, and the extent of the degeneration caused by the various grades of deficiency after various periods may be dealt with systematically.

*Diet B + 0 per cent. Yeast Extract.*—Owing to the fact that animals on this diet decline and die very rapidly only two stages in the degeneration of the testes of such animals were observed. P. 2, a buck, which had been on this diet 15 days and had lost 16 per cent. of its weight, was operated on and the right testis removed. Sections of this testis showed that in some of the tubules degeneration had proceeded to the stage where the disintegrated Sertoli tissue and germ cells form a sort of core in the tubule, while in other tubules apparently normal germ cells still persisted, and a few spermatozoa in the course of formation could be seen. Recovery from the operation was quite satis-

factory, but by the sixth week of dieting the animal was very thin and sickly and had lost 40 per cent. of its original weight. It was therefore killed and the left testis prepared and sectioned. Examination showed that nothing recognisable as spermatozoa was to be seen in any of the tubules, and that practically all the tubules showed the typical core of degenerative tissue. In a number of cases this core had disappeared, or was disappearing, to leave the almost empty tubule (fig. 5 of Allen's paper), which was in apparently the ultimate stage of degeneration (Plate 16, fig. 2) Eight animals in all were put on this diet, and all these either died or were killed just before death would have occurred, between the fourth and sixth weeks of dieting. Of the eighteen matings which were made during the second, third and fourth weeks, only two produced litters, one conception taking place in the second week and one in the fourth. Since the buck which fertilised a female in the fourth week died two weeks later, it would seem that a fairly advanced stage of degeneration, something between the two described above, is not incompatible with a limited fecundity.

*Diet B + 0.5 per cent. Yeast Extract* -- Sections illustrating two stages in the degeneration of the testes of rats in these diets were obtained. The testes of P. 113, which had been on the diet for five weeks, showed rather more advanced degeneration than that caused in the right testis of P. 2 by complete absence of vitamin B for 15 days. Very few spermatozoa were present and most of the tubules showed the characteristic blocking by tissue debris. The testes of P. 54 and P. 56, which were killed when dying in the seventh and ninth week respectively, showed very similar degeneration to each other, little more advanced than in the case of P. 113. P. 56 showed odd clumps of spermatozoa, rather less few in number than in P. 54. In odd tubules the final stage of degeneration had set in, but in no case was this very advanced. Of the eight animals put on this diet three were fertile in the third week, three, including P. 54 and P. 56, were fertile in the fourth week, and none later than this, the remaining two producing no litters. P. 54 and P. 56 had originally been large bucks, weighing respectively 365 gm. and 350 gm. when put on experiment. When killed P. 54 had lost 120 gm. or 32 per cent. of its original weight, and P. 56 had lost 138 gm. or 39 per cent.

*Diet B + 1 per cent. Yeast Extract.* -- Sections of the testes of animals on this diet were obtained for three periods of dieting. The first at 15 days, by unilateral castration (right testis) of P. 7, the second in the fourth week from the remaining testis of P. 7, and the third in the 13th week by killing P. 6. The right testis of P. 7 showed many tubules which had a tolerably normal appear-

ance, though in a few slight cores of debris had begun to form. The left testis when removed two weeks later showed greater degeneration, but there were still spermatozoa to be found. In the thirteenth week the testes of P. 6, though containing many tubules in early stages of degeneration (*see* Plate 16, fig. 3), still had spermatozoa, and this is well correlated with the fact that other animals in the group breed up to this time. P. 6 itself, though having no litters later, was found to be fertile in the seventh week. P. 6 had just maintained its weight, and P. 7 showed no loss except that attributable to the operation, and was in fact heavier when killed than when put on the diet.

One animal of this group is deserving of special mention. P. 5 had the right testis taken out at 15 days. This testis showed in many tubules normal spermatogenic activity, while in others some degeneration had set in. The animal was mated in the sixth week without producing a litter, and twice in the eighth week with the same result. From the tenth to the twentieth week, however, it produced litters regularly (six in all), and in this period, after having just maintained its weight up to the ninth week, increased its weight from 210 gm. to 280 gm. At the twenty-third week part of the remaining testis was removed as described above. This sample showed a remarkably normal appearance considering the time it had been on the deficiency diet. A typical section of this testis is shown in Plate 17, fig. 4, and large numbers of spermatozoa are to be seen. It is also worthy of note that the interstitial tissue shows hypertrophy. After this second operation, P. 5 was put on to natural food. The animal subsequently proved sterile, owing probably to the operated testis developing a tumour, which unfortunately also invalidated further histological examination. The case of this animal serves to emphasise the remarkable variation in resistance to vitamin B deficiency which characterises rats.

*Diet B + 2 per cent. Yeast Extract.*—Three stages of degeneration and a recovery stage were microscopically examined in this group. The right testis of P. 9 which was removed after 15 days dieting showed little pathological change and was practically normal. The left testis which was examined in the fourth week was still fairly normal, although a few tubules showed a small accumulation of debris in the lumen and were without spermatozoa. P. 9 lost no weight while on the diet. The ultimate stage of degeneration was obtained in three cases. P. 11 had the right testis removed after 23 weeks on the diet, and was then put on to normal food for the purpose of observing recovery. Testes were also taken from P. 12 (dying of diarrhoea at 15 weeks on diet) and from P. 64 (operated on at 18 weeks). None of these preparations show

any greater degeneration than the left testis of P. 9. P. 64 had lost weight while on diet, but the other two had actually gained. The right testis of P. 11 is shown in Plate 17, fig. 5 (a). Both P. 11 and P. 64 had proved fertile up to the fifth month. The left testis of P. 11 when examined nine weeks after the animal was put on normal food showed almost exactly the same appearance as the right testis. The left testis is shown in Plate 17, fig. 5(b). The inference is that as soon as degeneration has set in it is irreversible, although the animal may recover its weight and general health.

*Diets B + 3 per cent. and B + 4 per cent. Yeast Extract.*--The histological findings in both these classes were closely similar, and the two groups may therefore be dealt with together. Even after more than six months on the diets, only a very mild form of degeneration, debris in a very few tubules, was found. Plate 17, fig. 6 (a), shows this stage in the right testis of P. 15 (3 per cent. yeast). In both these classes the return of the buck to normal food does not seem to have improved the histological appearance of such tubes as had degenerated. The left testis of P. 17 (4 per cent. yeast) after nine weeks on normal food is shown in Plate 17, fig. 6 (b), and no great difference from P. 15 (right testis) is observable. This confirms the previous conclusion as to the absence of recovery as far as histological degeneration is concerned.

The fact that in these dietary groups so little degeneration takes place, that the animals grew well and were perfectly fertile the whole time, suggests that these diets supply an amount of vitamin approaching sufficiency

#### (4) *Fecundity.*

The term "fecundity" is here used to mean the proportion of matings resulting in pregnancy, while "fertility" is used solely in relation to the size of litter. The data relating to the number of matings producing young is summed up in the following table.---

Table I.--Fecundity according to degree of B Deficiency.

Diet.	Total Matings.	Total Litters.	Per cent. Pregnancies.
B Deficient	18	2	11 0
B + 0 5 per cent. yeast	25	9	36 0
B + 1        "	33	13	39 4
B + 2        "	44	17	38 7
B + 3        "	42	24	57 2
B + 4        "	17	10	58 9
B + 5        "	17	7	41 2
Normal	12	9	75 0
Animals from deficient groups recovered on normal diet	5	4	60 0
Total ..	213	95	44 6



From this table it will be seen that, generally speaking, the fecundity is very low in the complete deficiency group, but rises considerably in the group on diets only mildly deficient in vitamin B. The figure for bucks on diet B + 5 per cent. yeast is probably not an accurate representation of the facts.\* The fecundity is, naturally, highest among bucks on a normal diet of natural food.

It is thus possible to say that vitamin B deficiency lowers fecundity, and that the greater the deficiency the lower the fecundity. This result is exactly what might be expected. Long before the actual symptoms of beri-beri set in, the animal loses its vigour and tone, especially on the B and B + 0.5 per cent. yeast diets. In addition, microscopical examination showed that the spermatozoa had not normal vigour and mobility. It is reasonable to suppose, therefore, that the bucks in these groups were less capable of performing coitus, and that there was less chance of it proving effective. These results are shown graphically in fig. 1.

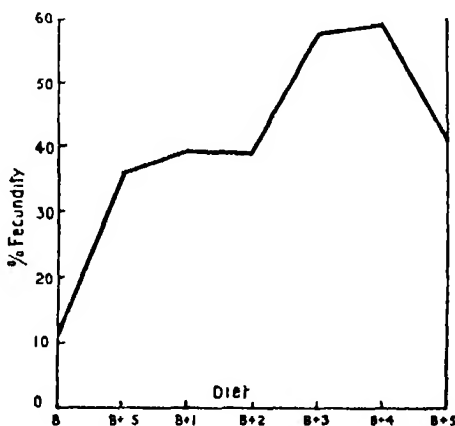


FIG. 1—Fecundity and diet.

#### (5) Fertility.

The high standard of fertility attained by these rats was one of the most remarkable features of the whole experiment. Long and Evans's (7) very extensive breeding data for rats shows only a fertility of 6.9; the following table shows the fertility attained by the different groups of dieted rats †

\* The B + 5 per cent. yeast bucks are not truly comparable with the rest of the series as they were originally part of another experiment, and had been subjected to rather different treatment. The young from these bucks will be omitted from further consideration.

† It should, of course, be remembered that in these experiments under discussion the females, who normally to a large extent govern fertility, were on natural diet.

Table II.—Fertility according to degree of B Deficiency.

Diet.	No of Litters Sexed.	No. of Young	Average
B	2	21	10 5
B + 0 5	8	56	7 0
B + 1	13	102	7 8
B + 2	15	97	6 5
B + 3	24	169	7 1
B + 4	10	76	7 6
B + 5	7	50	7 1
Recovered	4	31	7 7
Normal	9	68	7 9
Total	92	670	7 28

These results show that the degree of deficiency has no effect on fertility. Since a male, if fertile at all, can usually produce the largest litter that can be born by the female, this fact is not surprising.

It is possible, however, to look at the results of deficiency from another point of view, *i e*, to consider whether the length of time the animal has been on the diet has any results. The males on diets B and B + 0.5 per cent. yeast did not live long enough to make it possible to analyse their records from this angle, but in the B + 1 per cent. yeast a good series of animals were produced, and the deficiency is severe enough to have any effect possible. The following table gives the size of litters produced by B + 1 per cent. yeast bucks analysed by four-weekly periods.

Table III -- Fertility according to Time on Diet (B + 1 per cent.).

Time on Diet (Weeks).	No. of Litters.	No. of Young	Average.
1- 4	3	21	7 0
5- 8	—	—	—
9-12	5	40	8 0
13-16	2	16	8 0
17-20	3	25	8 3
Total	13	102	7 8

In so far as the numbers are significant, this table shows that the duration of the deficiency diet has no effect on the size of litter; again a finding in keeping with what might be anticipated.

In view of the good normal fertility which was found in these experiments, it is of import to consider the frequency distribution of litter and the resulting scatter of the series. Table IV gives the number of litters of each size.

Table IV.—Frequency distribution for size of litter.

Size of Litter.	No. of Litters	No. of Young.
1	—	—
2	2	4
3	2	6
4	4	16
5	4	20
6	9	54
7	19	133
8	19	152
9	13	117
10	2	20
11	5	55

For this series  $\delta^2 = 3.857$  and  $\delta = 1.96$ . This standard deviation is not large and is indeed rather less than that found for normal mice (Parkes 11). In any case it cannot be considered as showing any very abnormal features. As the females, which in these experiments were on normal diet, for the most part control fertility, this result is not unexpected.

#### (6) *Sex-Ratio* \*

The influence of deficiency upon the proportions of the sexes was one of the most important points of the experiments. The following table gives the numbers of males and females produced by the bucks on the various diets.

Table V —Sex ratio according to degree of deficiency

Diet	No of young	Males.	Females	Sex-Ratio	Probable error.
B +	21	11	10	52.4	—
B + 0.5	56	28	30	46.5	$\pm 4.49$
B + 1	102	41	61	40.2	$\pm 3.27$
B + 2	97	45	52	46.4	$\pm 3.41$
B + 3	169	82	87	48.5	$\pm 2.12$
B + 4	76	37	39	48.7	$\pm 3.86$
B + 5	50	26	24	52.0	$\pm 4.76$
Normal	68	34	34	50.0	$\pm 4.09$
Total	639	302	337	47.3	$\pm 1.29$

With minor exceptions this table suggests that there is a progressive decrease in the proportion of males as the degree of vitamin deficiency increases, but

\* Calculated in all cases as percentage of males, of which the probable error can be calculated from the formula  $0.6745 \sqrt{mf/n}$ , where  $m$  and  $f$  are the proportions of males and females and  $n$  the number of young.

in each case the probable error of the percentage of males is large. In fact, the only ratio which is significantly different from the normal is that for the offspring of males on B + 1 diet. In the following table are given the differences from the normal, the probable errors of the differences, and relation of the two.

Table VI — Probable errors of differences from normal

Diet	Difference of ratio from normal	Probable error of difference *	<u>Difference</u> Error
B + 0 5	3 5	6 07	0 577
B + 1	9 8	5 23	1 87
B + 2	3 6	5 32	0 076
B + 3	1 5	4 60	0 326
B + 4	1 3	5 62	0 231
B + 5	2 0	6 20	0 323

\* Calculated from the formula  $\sqrt{A^2 + B^2}$ , where A and B are the probable errors of the two ratios to be compared

Little importance can, therefore, be attached to these ratios as regards individual dietary categories. It is possible, however, to consider the diets as falling into two groups, those with 3 per cent. of yeast extract and over, which have an amount of vitamin B approaching sufficiency, and those with 2 per cent. or less, which are definitely deficient in vitamin

When the results are grouped according to this classification the figures are as follows :

Diet.	No. of young	Males	Females	Sex-Ratio	Probable Error
B + 2 and less	276	123	153	44 6	$\pm 2 02$
B + 3 „ more	295	145	150	49 2	$\pm 1 96$

The definitely deficient group gives a sex-ratio of  $44.6 \pm 2.02$ , which is beyond the normal range, and the less deficient group gives one of  $49.2 \pm 1.96$ , which cannot be considered abnormal. The probable error of the difference between these two ratios =  $\sqrt{2.02^2 + 1.96^2} = 2.81$ . As the actual difference is 4.6, it is 1.64 times its error, and is therefore significant, but not sufficiently so to carry much weight.

Considering the variation in the sex-ratio from the point of duration of dieting the following results were obtained.

Table VII.—Sex-ratio according to degree of

Diet	Weeks									
	2	3	4	6	7	8	9	10	11	12
B + 0	♂ 4 ♀ 6	♂ — ♀ —	♂ 7 ♀ 4	♂ — ♀ —	♂ — ♀ —	♂ — ♀ —	♂ — ♀ —	♂ — ♀ —	♂ — ♀ —	♂ — ♀ —
B + 0.5	—	17 15	9 15	—	—	—	—	—	—	—
B + 1	—	—	12 9	—	—	—	1 8	4 2	2 6	5 12
B + 2	—	—	10 9	—	1 1	10 14	—	4 5	—	4 3
B + 3	—	—	—	35 36	—	—	—	7 5	—	—
B + 4	—	—	—	—	—	—	3 1	—	—	9 8
Totals	4 6	17 15	38 37	35 36	1 1	10 14	4 9	15 12	2 6	18 23
Total	10	32	75	71	2	24	13	27	8	41
Monthly Totals	♂ 59 ♀ 58		♂ 46 ♀ 51		♂ 30 ♀ 50					
Monthly Total	117		97		80					
Monthly Sex-ratio	50.4 ± 3.12		47.4 ± 3.42		44.0 ± 3.55					

A consideration of this table seems to suggest that the proportion of males declines both with increasing deficiency and with lengthening of time on the diet. The 79 births resulting from conceptions by bucks in the fifth month of dieting give only 32 males, a percentage of 40.5, while births from conceptions in the first month give a percentage of 50.4, normal to all purposes. It should also be noticed that conceptions in the first month are mainly from the very deficient bucks. It would appear, therefore, that for the first month dieting has no effect on the sex-ratio. An interesting fact emerges if we exclude conceptions in the first month from the totals according to degree of deficiency. The figures (see Table VIII) then run as follows :—

deficiency and duration of Dieting.

on Diet.								Totals	Total
13	14	15	16	17	18	20	24		
♂   ♀	♂   ♀	♂   ♀	♂   ♀	♂   ♀	♂   ♀	♂   ♀	♂   ♀	♂   ♀	
—   —	—   —	—   —	—   —	—   —	—   —	—   —	—   —	11   10	21
—   —	5   4	3   5	—   —	6   10	—   —	3   4	—   —	26   30	56
5   4	—   —	6   8	3   4	2   5	—   —	—   —	—   —	41   61	102
—   —	11   7	—   —	—   —	4   11	1   1	6   7	18   20	45   52	97
—   —	—   —	6   11	9   10	—   —	10   9	—   —	—   —	82   87	169
5   4	16   11	15   24	12   14	12   26	11   10	9   11	18   20	37   39	76
9	27	39	26	38	21	20	38	242   279	521
♂   ♀ 18   53				♂   ♀ 32   47			♂   ♀ 18   20	—	
101				79			38	—	
47.5 ± 3.34				40.5 ± 3.72			17.5 5.45	—	

Table VIII.—Sex-ratio of conceptions after the first month according to degree of deficiency.

Diet.	Males	Females	Total	Percentage Males	Probable error
B + 1	29	52	81	35.8	± 3.59
B + 2	35	43	78	44.9	± 3.90
B + 3	82	87	169	48.5	± 2.12
B + 4	37	39	76	48.7	± 3.86
Normal	34	34	68	50.0	± 4.09

The relation of the differences from the normal and the probable errors of the differences are shown in the following table (Table IX).

Table IX.—Differences from normal with probable errors of differences from Table VIII.

Diet	Difference from normal	Probable error of difference.	$\frac{\text{Difference}}{\text{Error}}$
B + 1	14.2	5.44	2.61
B + 2	5.1	5.58	0.914
B + 3	1.5	4.60	0.326
B + 4	1.3	5.62	0.231

These tables show that the decrease of the male percentage is much more noticeable when the conceptions of the initial month are excluded. In fact the conceptions by bucks in the later stages of B + 1 per cent. marmite deficiency produced nearly two females per male, and the difference from the normal is 2.61 times its probable error, and is therefore well significant.

Turning, however, to a more detailed survey of the relation between sex-ratio and time on diet, a very obvious decline is noticed in the proportion of males among litters conceived after two months of dieting. This decline may be expressed graphically.

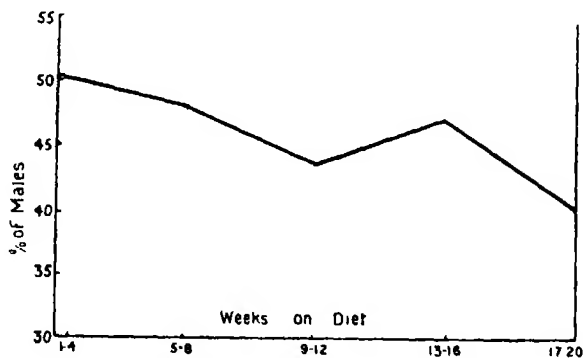


FIG. 2 —Time on Diet and Percentage of Males produced.

The 38 young produced as the result of conceptions more than five months after the beginning of dieting may be neglected owing to their paucity of numbers.

If the 20-week period be divided into two sections of 8 weeks and 12 weeks, respectively, the following results emerge :—

\* Table X. Grouping of Table VII.

Period.	Males.	Females	Total	Sex-Ratio	Probable error
1-8 weeks	105	109	214	49.1	$\pm 2.31$
9-20 "	119	150	269	14.2	$\pm 2.04$
Total	224	259	483	46.3	—

The probable error of the difference between the percentage of males in these groups is  $\sqrt{2.31^2 + 2.04^2} = 3.08$ . Hence, the difference is  $4.9 \pm 3.08$ , i.e., it is more than 1.6 times its error, and is therefore significant.

It will be remembered that the figures given in this same section relating to sex-ratio and degree of deficiency show much the same result for the two dietary groups, and it may be concluded that the proportion of males tends to drop both with increasing deficiency and with prolonged dieting. Possible explanations of the results will be discussed in a later section.

#### (7) *Weight of Young at Birth.*

It was, unfortunately, found impracticable to obtain the growth curves of the young sired by the bucks on B deficiency diets. The weight at birth, however, was usually noted, and the following table gives the results:—

Table XI — Weight of Young at Birth.

Diet	No. of young weighed	Total weight. (grms.)	Average weight (grms.)
B + 0.5	48	274	5.7
B + 1	116	623	5.4
B + 2	97	495	5.1
B + 3	128	712	5.6
B + 4	20	104	5.2

Since Donaldson (2) gives the average weight at birth as 5.6, these weights cannot be considered to be abnormal. It was noticed, however, that the young born in the deficient groups did not show normal growth, and that they were subsequently undersized.

#### (8) *Discussion.*

It is proposed to discuss here the results recorded upon under their respective headings.



*Fecundity and Fertility.*—The fact that fecundity decreased steadily with decreasing vitamin content of the food (*see* Table I) might be due to either or both of two reasons—(a) lack of vigour on the part of the male, (b) decreased vitality of spermatozoa. As regards the first, animals on diets badly deficient in vitamin B become extremely lethargic long before the fatal sharp decline in weight and the beri-beri symptoms set in, and such debility of the animal must lead to a decline of the sex desire. The question of the vitality of the spermatozoa was gone into more accurately. Since the collection of the seminal fluid of the rat is hardly practicable, the method employed was to tease out the epididymis into mammalian Ringer's solution at 37° C., and to examine microscopically. This was done during the post-mortems made on animals killed\* at varying period before deficiency death would have occurred, and in some cases actually during what would have been the fatal seizure. In this way a check upon the condition of the spermatozoa was obtained. Rat P. 54, which had been on a B + 0.5 per cent yeast extract diet for 47 days, and which had lost more than 30 per cent. of its original weight during this time, was killed when showing acute beri-beri symptoms, and was found to have general stagnation of the venous system, and especially of the spermatoc vessels, the tunica of the testes, in particular, having a peculiarly "blood-shot" appearance. The epididymis contained a large number of dead mature spermatozoa, and a teased preparation of the testes showed large numbers of dead immature spermatozoa. The buck had fertilised a doe after being on diet 27 days, though subsequent matings failed, as had two matings after being on diet 13 days.

P. 55, another of the same group of animals, was killed two days later, before the beri-beri symptoms became acute and exhibited a similar condition, except that fewer spermatozoa (all dead) were found. This animal had lost nearly 50 per cent. of its weight. The same animal had fertilised two females after being dieted for 27 days, but both litters were born dead. A subsequent mating produced no results.

P. 56, another animal of this group, which had lost 40 per cent. of its weight, was killed, after 64 days' dieting, just before death would have occurred. This animal, however, must have had a greater tolerance of the deficiency, because the stagnation of the vascular system was not pronounced and large numbers of fully active spermatozoa were found in the epididymis. P. 56 fathered a good litter after having been on the diet 30 days, but subsequent matings

\* Decapitation was employed to kill the animals, since over-dosing with chloroform or ether kills the spermatozoa as well.

failed. P 6, which had been on a B + 1 per cent. yeast extract diet for 59 days but lost very little weight, was found to possess large numbers of active spermatozoa. This animal was fertile up to 26 days but not after P. 58. On the other hand, which had been on the same diet for only 49 days but had lost 45 per cent of its weight, had comparatively few spermatozoa, of which only a fraction were active. This buck fertilised a doe after being on diet 30 days, but the litter produced was a very poor one.

Two other males, from the B +2 per cent yeast-extract group, were examined in this manner. P. 12 which had just maintained its weight after being on diet for 79 days, showed no serious venous stagnation and the testes were fairly normal. Plenty of spermatozoa, mostly active, were observed in the epididymis, but none of its four matings had been successful. The other animal examined from this group, P. 62, had been on diet 90 days when killed, and had lost 30 per cent of its weight in this time. Vascular stagnation was very marked, and the testes were bluish and "bloodshot". The spermatozoa were inactive. It had fertilized two does, the last one after being on diet 49 days. At 70 days it was completely sterile.

These observations explain the results of the deficiency diets on both fecundity and fertility. Though much individual variation was found, it is clear that the deficiency affects the vitality of the spermatozoa, and therefore lowers the fecundity. At the same time, however, if a buck is capable of fecundating a doe at all, it is just as capable of producing a normal-size litter as of producing one of, say, three. This is emphasized by the fact that the standard deviation is quite a normal value.

*Sex-ratio.*—The alteration of the sex-ratio produced by vitamin B deficiency must be discussed in the light of the two factors which are known to govern the sex-ratio at birth. These two factors are : (a) the sex-ratio at conception, (b) the amount and sex incidence of the foetal elimination which precedes birth. In many cases it has been found that the pre-natal mortality which occurs may have a very considerable influence upon the nature of the ratio at birth. In many mammals, including mice (10), it has been shown that pre-natal mortality falls predominate upon the males, and that a large amount of foetal death results in a considerable decrease in the proportion of males at birth. Since in most mammals there is normally a small excess of males at birth, and since also there is in all cases a very appreciable amount of mortality during gestation, the excess of males at conception must be fairly considerable. At first sight this fact appears to conflict with the chromosome theory of sex determination, which theory is now generally accepted. It is probable, however,

that no real contradiction is implied. Granted that the male and female-producing spermatozoa are produced in equal numbers, it does not necessarily follow that they arrive in the Fallopian tubes of the female in a similar proportion. Their competitive existence in the epididymis and their competitive passage to the ova, provide an ample basis for the operation of selective factors, and the presence or absence of an extra chromosome may be a further characteristic upon which selection may act, and if this is so, selection would be between the male-producing and female-producing types.

The two chief results of the effect of vitamin B deficiency upon the sex ratio which have to be discussed here are: (a) that a deficiency represented by 2 per cent or less of yeast extract in the food has an effect upon the ratio, and that this effect is more marked after the first month of dieting; (b) that the proportion of males also decreases with the duration of the dieting. The question is, to which of the two factors governing the ratio at birth is this result to be attributed? In the first place, we may deal with the possibility of pre-natal mortality. This factor does not seem to be the probable one. Firstly, the females used in these experiments were perfectly normal, both in diet and in constitution, and, secondly, the very normal fertility found argues nothing very abnormal in the amount of elimination. It should be mentioned, however, that Hammond (6) has shown that in certain cases an increased amount of pre-natal mortality, which would produce the results obtained, may be brought about by debility of the male, and hence of the spermatozoa. In some experiments carried out in confirmation of the work of Evans and Bishop (4), where both male and female were kept on diet deficient in the hypothetical vitamin X, it was noticed that such young as were produced showed a most striking excess of females. Since the cause of the partial sterility in this case is due to the great frequency of foetal re-absorption, this shortage of males may be put down to excessive wastage before birth. This possibility does not appear to be of any great importance in the present case when weighed against the evidence which seems to exclude pre-natal mortality as the explanation of the present results.

We are thus reduced to seeking the cause of the observed results in some alteration of the ratio at conception, and it would appear that the deficiency of an essential substance has reacted more unfavourably upon one type of spermatozoa than upon the other. That the deficiency does react unfavourably is shown by the lower fecundity of dieted males and an extension of this concept is not unreasonable. The most important objection to this idea is that a certain amount of other work suggests that the effect of bad conditions on the sperma-

tozoa is to upset the balance in favour of the male-producing spermatozoa and therefore to produce an excess of males, not an excess of females, such as we are dealing with here. There seems, however, no alternative explanation to the observation that any serious deficiency of vitamin B disturbs the sex-ratio at birth and presumably the sex-ratio at conception.

Why the proportions of males should also fall with the duration of the dieting is a still more difficult problem. There are three possible ways in which this could be correlated with the decrease due to actual graded deficiency in the diet. Firstly, the facts would be accounted for if any considerable storage of vitamin B took place, since as time went on the storage would become exhausted and the same results would be produced as by deficiency in the diet. There is, however, much evidence to show that practically no storage of vitamin B can take place. Growth, for instance, ceases almost immediately that the external supply of this vitamin is stopped. Storage, therefore, cannot be the required explanation. The second possibility is that the tissues have the capacity to carry on, though with decrease in vigour, for a certain time without an adequate supply. The fact that animals which are entirely minus an external supply do not die immediately but gradually decline, seems to show that this is so, but it is hard to see how this could apply to a transient body like the spermatozoon. The most probable explanation is that an actual increasing deficiency, as time goes on, is brought about by the fact that dieted animals tend to eat less and less of the synthetic food, and that they are therefore taking in a constantly less supply of the vitamin, and that they are therefore virtually on a greater deficiency each month. In this way the time relation would be correlated with the degree of deficiency relation, and the observed results would be produced.

#### (9) *Summary.*

(1) When a buck rat is put on a diet totally deficient in vitamin B, degeneration of the testes and sterility ensue in a short time.

(2) On diets only partially deficient in the vitamin these results may be postponed temporarily or indefinitely according to the acuteness of the deficiency.

(3) The amount of degeneration of the testes can be correlated both with the degree of deficiency and with the time on the diet, but owing to the great variation found from tubule to tubule in the same testes this correlation is only general. Once the degeneration has become severe, normal dieting, though restoring the body weight and vigour of the animal, does not result in a return to normality in the testes.

(4) The fecundity (*i.e.*, fecundating power) of the buck can be definitely correlated with the degree of deficiency, severe deficiency resulting in a greatly lowered fecundity even before complete sterility supervenes.

(5) The fertility (*i.e.*, size of litter), however, shows little variation. A buck, if capable of reproducing at all, is apparently capable of fertilizing as many ova as the female can develop.

(6) The proportion of males among the young decreases both with the degree of deficiency and with the time on diet, a finding which is probably explained by the fact that as the animals become tired of the diet they eat less, and thus, for practical purposes, have a constantly decreasing supply of vitamin.

(7) The reason why a deficiency of vitamin should lower the proportion of males, together with possible explanations of the other results obtained, are briefly discussed.

The expenses of the investigation described above were defrayed from a grant made to one of us (J.C.D.) by the Medical Research Council.

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#### DESCRIPTION OF PLATES 16, 17.

##### PLATE 16.

FIG. 1. (a) Early stage of core formation in lumen of tubule from tissue debris.

(b) Later stage, with lumen completely obliterated.

(c) Ultimate stage of degeneration in tubule, showing tubule almost emptied of contents.

FIG. 2. Testis from male (P. 2) dying of beri-beri after complete vitamin B deficiency for 4 weeks, showing ultimate degeneration.

FIG. 3. Testis of P. 6 after 13 weeks on diet B + 1 per cent. yeast extract, showing some of the tubules in which degeneration has just commenced.

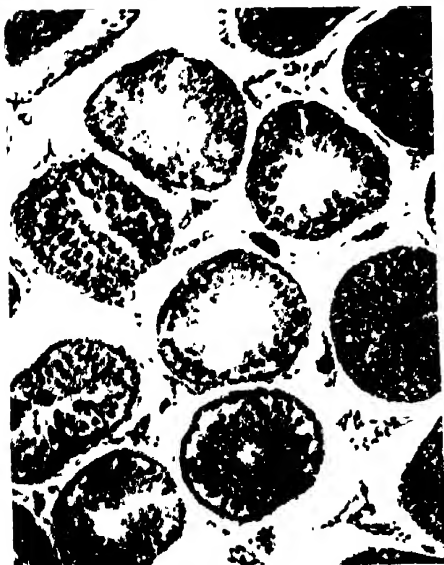
I a



I b



I c

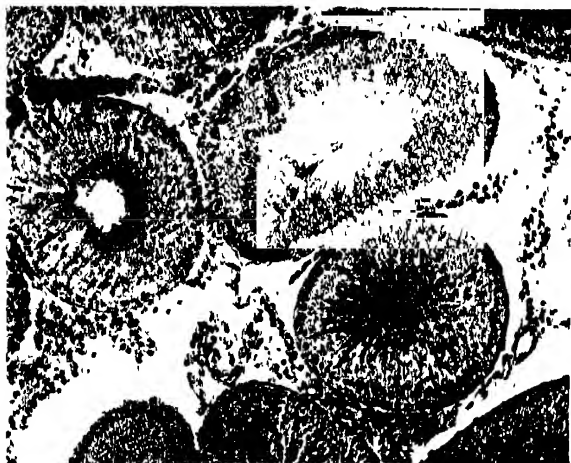


II



III

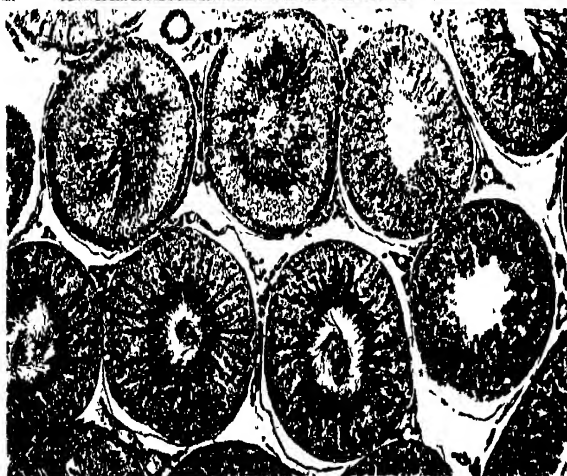
IV



V b.



V a



VI b



VI a



PLATE 17.

- FIG. 4. Testis of P. 5 after 23 weeks on diet B + 1 per cent. yeast extract, showing numbers of spermatozoa and hypertrophy of interstitial tissue.
- FIG. 5. (a) Right testis P. 11 after 23 weeks on diet B + 2 per cent. yeast extract, showing absence of serious degeneration even after long periods on this diet.  
(b) Left testis of same animal after 9 weeks on normal food.
- FIG. 6. (a) Right testis P. 15 (3 per cent.) yeast showing the very slight degeneration which occurred even after 27 weeks on the diet.  
(b) Left testis P. 17 (4 per cent. yeast), after 9 weeks recovery on normal diet.

The magnification of fig. 1 is 210, and of the other figs. 120.

The separation of the tubules from each other is caused by the fact that the testes were cut up into very small pieces for fixing, and therefore a tendency to disintegrate was inevitable.

*Studies on the Biological Action of Light.*

By D. T. HARRIS.\*

(Communicated by Prof A. V. Hill, F.R.S —Received January 29, 1925.)

(From the Physiology Institute, University College, London.)

[PLATE 18.]

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SECTION I.—THE EFFECT OF LIGHT ON THE METABOLIC RATE OF SMALL ANIMALS.

*Introduction.*—Whereas plant life is directly dependent on the supply of radiant energy from the sun or an artificial source of light, animals may lead perfectly healthy lives in complete darkness. Early observations by K. A. Hasselbalch on the total metabolic changes in experimental animals, and by A. Durig and his co-workers on the respiratory exchange in man, seemed to show that light was without action on animal metabolism. Recently, however, the

\* Working for the Biological Action of Light Committee of the Medical Research Council.



therapeutic application of sunlight at Alton and Leysin (1) and the radiations of the large carbon arc in the hospitals of our large towns have yielded results which suggest a stimulant action on general metabolism. Measurements by Leonard Hill and A. C. Campbell (2), however, show that the open-air conditions were mainly responsible at the former resorts. The present investigation of the gaseous metabolism of small animals supplies an approximate measurement of the total metabolism during exposure to artificial radiations.

*Method.*—The rat was the species chosen as it could be comfortably confined in a small quartz beaker used as a respiration chamber. The latter was fitted up in a manner somewhat similar to the arrangement in the Haldane-Pembrey respiration apparatus.

The radiations employed were those emitted either from a large quartz mercury-vapour lamp ( $\frac{1}{2}$  kw) or from a small carbon or metal arc ( $\frac{1}{4}$  kw). For testing the effect of visible light only, either a quartz beaker with a plate-glass filter or a glass beaker was used. For the ultra-violet radiations blue uviole glass with a range of transmissibility from 291 to 436  $\mu\mu$  (see fig. 1, Plate 18) was used to filter off the long visible rays. Fig. 1 also shows quartz mercury-vapour emission spectrum.

The temperature of the respiration chamber was kept constant or quickly varied by means of either a cold-water spray encircling the beaker or heated black material. Estimations were not registered until the animal had settled down; a quiet condition was soon reached when a sufficient supply of air was established. Absorbing tubes were then selected, large enough to trap all the carbon dioxide with this mode of aeration, and these were coupled to a second set of tubes as a control.

A considerable number of preliminary estimations were found necessary in the adjustment of the size of the absorbing tubes, the duration of a single observation, and the rate of aeration, for the purpose of establishing constancy of the conditions for a given size of rat. All cases in which the rat exhibited unusual movement, etc., were rejected; usually the animal fell asleep. At first, the oxygen intake as well as the carbon dioxide output were estimated, but the determination of the former was abandoned as it could not be obtained with the same degree of accuracy as the carbon dioxide. This was unfortunate in view of the just criticism of the method of using the carbon dioxide only, made by Cathcart, Leonard Hill and Campbell, and others; but the method of continued quarter-hourly observations in the experiments to be described remove most of the causes for objection to this decision, especially in view of the fact that whereas the carbon dioxide could be weighed with an accuracy of

$\pm 0.5$  per cent., the *quarter-hourly* oxygen by the method used possessed an error of no less than  $\pm 25.0$  per cent.

*Control Experiments.*—The suitability of these small animals for the experiments was tested by a series of preliminary determinations. Since a restful state of the animal was essential the regular feeding of the animals in their cages was adhered to, and it was soon found that the animal settled down immediately if it were trained gradually for three periods on successive days to accommodate itself to the respiration chamber.

The rate of fall in the basal metabolism of various rats and mice exhibited a constancy for a length of time varying with the size of the animal; after this time the animal showed marked fluctuations, due to restlessness arising from thirst. Examples are presented in fig. 2, in which the ordinates denote

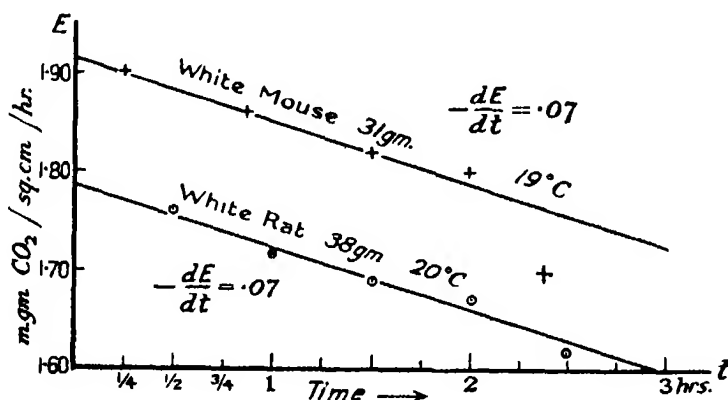


FIG. 2—Metabolic rate in darkness.

the rate of carbon dioxide production per unit of surface, the latter quantity being obtained from the weight  $W$  of the animal by using the approximate formula  $10 \times W^{\frac{1}{2}}$  applicable to small rats and large mice.

The immediate effect of switching the light on to a resting rat is to excite washing operations, which send up the metabolic rate very slightly. Conflicting results were obtained in the earlier experiments until all causes of movement (*e.g.*, projections into the respiration chamber, which might provide ends for nibbling) were eliminated and until unceasing observation yielded a constancy in temperature.

No evidence was found for an action of the mercury vapour radiations on the air breathed. Air drawn from the immediate vicinity of the lamp was led, after purification into an irradiated quartz beaker communicating directly with a glass bottle in which the animal was placed. The following are averages

of the results of two experiments on two rats of nearly the same size over nine quarter-hourly periods.---

Rat.	Air T°	Mgm. CO <sub>2</sub> output when breathing.	
		Room Air	Irradiated Air.
White	20°	57.0	56.8
Black	20°	55	54

Such results lend no support to the views of Kestner, but are in agreement with those of Webster and Leonard Hill, and with the more recent findings of Hume and Smith (3) on the absence of any growth-promoting factor in irradiated air.

*Effect of Radiations of Different Wave-lengths*—The full radiations of the mercury-vapour lamp are without appreciable effect—other than that due to a slight rousing on the carbon dioxide output, as is seen in the following typical experiment (nine observations) carried out on a white rat weighing 48.3 grams at the beginning of the experiment. The respiration chamber was kept at a temperature of 17° C., and observations were made every quarter of an hour

	Mgm. CO <sub>2</sub> .
Darkness	75
Visible rays (Hg lamp/glass)	79
Visible and ultra violet (Hg lamp/quartz)	79

When the ultra-violet radiations alone ( $\lambda = 291 - 436 \mu\mu$ ) were employed, surprising results were obtained. In a series of four white rats exposed to those radiations of the quartz mercury-vapour lamp ( $\frac{1}{2}$  kw. at 25 cm.) transmitted by a screen of blue uviol glass previously placed in position, the following figures were arrived at by arranging an exposure in the middle third of the constantly-declining portion of the curve (*see fig. 2*) and interpolating for the control —

	Mgm CO <sub>2</sub> /l hr				Average.
Ultra-violet	65	77.5	65	56	66
Dark control	53	68.0	53	48	55

(Temperature of respiration chamber 19° C. in second case, 28° in all the others)

Contrary to the expectation that the non-luminous nature of these radiations would have a non-rousing character, we see that they possess a stimulant action on gaseous metabolism of appreciable magnitude—larger than can be ruled out by experimental variations.

A similar relation holds between the action of artificial ultra-violet rays and the action of diffuse daylight, *e g*, in the two light-grey rats exposed to the filtered radiations of the iron arc ( $\frac{1}{4}$  kw. at 15 cm.) :—

	Mgm. CO <sub>2</sub> / $\frac{1}{4}$ hr.	
Ultra-violet	81	81
Diffuse daylight	65	69

(Respiration chamber, 20°)

The difference is of the same order of magnitude as in the previous experiments

*Antagonising Effect of Long and Short Wave-lengths* —The evidence available relating to the action of light on the metabolic rate in man is of a conflicting character. On the one hand, clinicians maintain that insolation has a very definite stimulating effect on metabolism, whereas, on the other hand, actual measurements by different observers point to the absence of any appreciable difference in metabolic rate (2).

With the use of small animals, the experimental conditions can be more completely controlled. While observing the precautions outlined in the experiments of the previous paragraphs, the following figures for the gaseous metabolism were obtained under the action of ultra-violet radiations alone and under the action of ultra-violet admixed with luminous radiations : —

	Mgm. CO <sub>2</sub> / $\frac{1}{4}$ hr							
Ultra-violet only	78	56	81	59	79	85	79	64
Naked arc	59	48	67	47	73	80	73	54
Dark control	58	48	67	49				

(The source of light was the Fe arc in the last case, the Hg arc in all others)

These results show the nullifying effect of adding the visible radiations to the ultra-violet. While these experiments were in progress, Hess, Unger and Pappenheimer (4) published their discovery of the action of the visible rays in interfering with the curative effect of ultra-violet radiations ( $\lambda = 310 \mu\mu$ ) in rickets. Mme. and M. V. Henri had previously observed that the slowing of

protoplasmic movement by ultra-violet radiations was much less if illuminated by visible light at the same time (5). More recently, L. Hill (6) has shown that the immobilizing effect of ultra-violet rays on infusoria could be considerably reduced by the mere addition of red light. It is interesting to note in this connection the purely physical phenomenon of the power of quenching phosphorescence possessed by red and infra-red rays (7).

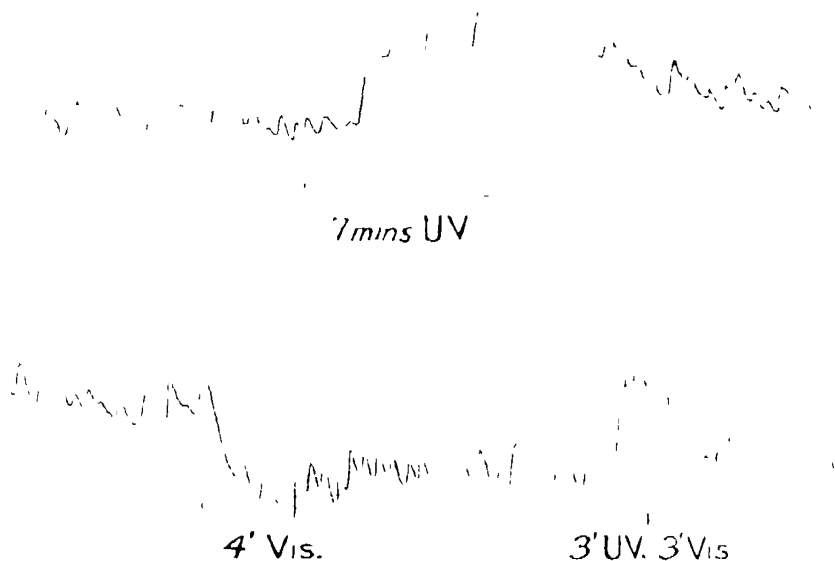
Herein we have a possible explanation of the absence of any measurable effect of mixed radiations on the gaseous metabolism in the estimations carried out by earlier workers, also of the freedom of human beings exposed to direct sunlight from any deleterious action of ultra-violet rays, in virtue of the nullifying action of the luminous radiations.

The frog's stomach, which had been found by Babkin to be an exceptionally photo-sensitive organ, had, nevertheless, yielded inconstant responses; it was therefore decided to investigate this irregularity on the lines of the present section. The isolated stomach was attached to one arm of a light lever; the stomach was kept alive in oxygenated Ringer's solution contained in a large rectangular trough, with one side of thin quartz and the opposite side of crown glass. An iron arc ( $\frac{1}{4}$  kw.) was used as a source of light; the heat rays were filtered off by a double uviol glass cell containing distilled water. The distance of the arc with the filter interposed was found to be an important factor in eliciting the effect to be described (15 cm. in these experiments). No rise of temperature was recorded on a thermometer reading to  $0.1^{\circ}\text{C}$ . placed in the saline medium bathing the stomach.

Wide variations in the nature of the response to radiations are manifested by different frogs and in different seasons, but the curves shown in fig. 3 present the typical form and were repeatedly obtained. The first curve (fig. 3, i) shows the marked increase of tone due to a seven minutes' exposure to ultra-violet radiations ( $\lambda = 291 - 436 \mu\mu$ ) obtained by means of a blue-uviolet plate (fig. 1). In the second curve (fig. 3, ii) can be seen the opposite effect, namely, a relaxation. The third curve (fig. 3, iii) is interesting in showing the immediate reversal of the ultra-violet effect when the blue-uviolet filter was removed, thus exposing the stomach to the visible as well as to the ultra-violet radiations; it is quite unlike the gradual decline seen in fig. 3, ii, when the ultra-violet radiations are merely extinguished. For this reason, it must be supposed that the phenomenon is not one of physical interference, even though the vibration frequencies of the ultra-violet radiations employed corresponded approximately with the octave of the visible. Whereas the reinforcement of the ultra-violet by visible radiations precipitates a steep fall in the third curve, the mere shutting

off of the ultra-violet is followed by a very gradual return to the normal state of tonus as seen in the first curve. It appears from these tracings that the

i. Seven minutes' exposure to ultra-violet radiations.



ii. Four minutes to visible light.

iii. Three minutes to ultra-violet and three to visible light.

FIG. 3.—Frog's stomach in oxygenated Ringer's solution; spontaneous contractions in darkness and the effect of light.

phenomenon is not one of physical interference, otherwise the fall in fig. 3, iii, would correspond with the decline in fig. 3, i; it is obvious, also, that the quantity of energy emitted is not the deciding factor. Thus ultra-violet radiations constitute an adequate stimulus to the frog's stomach while visible radiations exert an antagonising effect, which must be regarded, for the present, as a purely physiological phenomenon.

The results of the two different types of experiments point to the possession of a tonic action by ultra-violet radiations which is wiped out by combining with it the antagonising action of the visible radiations. In countries where artificial sources are employed in light therapy, the mercury arc is usually reinforced with ordinary filament lamps to complete the gaps in the spectrum

of the mercury vapour. This practice is justified on a purely physical basis regardless of the physiological actions of the different radiations.

Clinical tests are now being undertaken by the writer to determine the relative curative action of ultra-violet alone, the visible alone, and the composite actions of the ultra-violet and visible radiations.

### *Conclusions.*

1. Ultra-violet radiations exert a stimulant action on the gaseous metabolism of small animals and on the movements of the isolated stomach of the frog.

2. The stimulant action of ultra-violet radiations is completely annulled by the presence of visible radiations.

3. The latter phenomenon is one of physiological antagonism rather than physical interference.

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## SECTION II.—THE PART PLAYED BY PIGMENT IN THE SKIN OF ANIMALS.

*Introduction.*—The usually accepted hypothesis regarding the protection afforded by pigment in the negro's skin against the tropical sun (1) is one involving the *physical regulation* of body temperature by cutaneous hyperæmia and sweating thus constituting a process of *heat loss*.

During the prosecution of experiments on the metabolic rates in animals exposed to various radiations, it was noted that different coloured rats subjected to the same elevation of temperature exhibited different degrees of reduction in energy expenditure in such a manner as to suggest that pigment is an agent in reducing *heat production* which is a *chemical* process in the regulation of body temperature.

*The Protective Action of Pigment against Insolation.*—In view of the high absorption coefficient of the melanin pigment of the skin (2), the former hypothesis is more easily harmonised with the physical phenomenon of the absorption of radiant energy by melanin. A simple explanation would then appear to meet the case: radiations would be prevented by the pigment from penetrating the subcutaneous tissues and would then cause an intense heat stimulation of the epidermis and the closely neighbouring heat receptors, giving rise to reflex sweating. Although this may undoubtedly take place to some extent, quite a different type of mechanism appears to be brought into play in the experiments about to be described on the effect of exposing albino and pigmented rats to the same rise of temperature.

The experimental arrangement was precisely the same as that employed in Section I of this paper. The method of arriving at the data for the comparison of different animals may be seen from fig. 4.

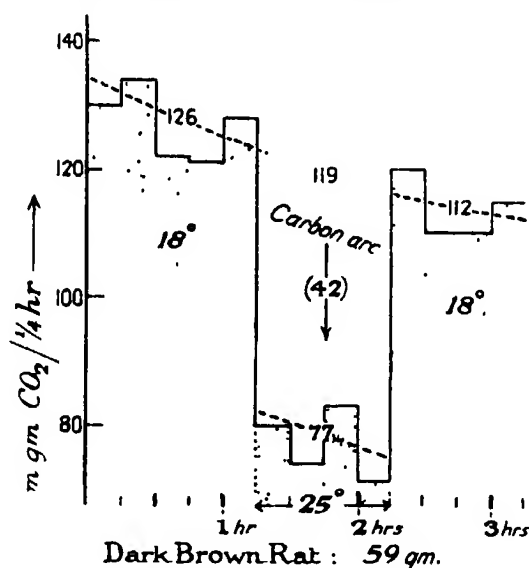


FIG. 4.—Lowering of metabolic rate by rise of temperature.

On raising the air temperature, as recorded by a shielded thermometer, from 18° to 25° C., which usually took from two to three minutes, there was a drop in the energy expenditure to 77 in the mid-period of the four 1/4-hourly observations. Now, at this time, if the temperature instead had remained constant, the energy expenditure at the mid-period would have been 119 (by interpolation) for 18° C. Thus, for a rise of temperature of 7° there occurred a drop in energy expenditure corresponding to 119-77 or 42 mgm. CO<sub>2</sub>.



The protocols of complete comparisons appear as follows :—

Effect of Pigment on the Metabolic Rate during a Rise of Temperature  $\theta$ .

$$\theta = 25^{\circ} - 18^{\circ} = 7^{\circ} \text{ C.}$$

Rat.	Weight W Grams.	Surface area S Sq. Cm.	Radiations.	Fall in CO <sub>2</sub> produc- tion — E	Rate of fall per sq. cm. per rise of 1° C. — E/S $\theta \times 10^4$	Extra lowering due to Pigment.
						Per cent.
White	67	165	Hg/quartz	{ 26	22.5	16.8
Black	61	155		{ 41	37.8	
White	83	190	Hg/blue-violet	{ 45	33.9	16.3
Black	64	160		{ 64	57.0	
White	67	165	Carbon arc	{ 40	34.5	14.5
Black	59	152		{ 42	39.5	
White	83	190	Dark heat	{ 51	38.4	13.0
Black	56	146		{ 51	50.0	
White	54	143	Dark heat	{ 22	22.0	11.8
Black	54	143		{ 26	26.0	

Undoubtedly the experimental procedures on a normal living animal open up a wide range of possibilities for variations in the above estimations, but the regular appearances of differences of the order of 10 to 15 per cent. allow the following deduction to be drawn: Pigment enables an animal, when subjected to a raised temperature, to reduce its energy expenditure far more (14½ per cent. more on the average, in the experiments quoted above) than if the animal were unpigmented. The uniformity of the results—within the range of experimental variation—whatever the source of radiant energy employed would suggest that pigment causes the skin to be hotter at a given external temperature, and therefore causes a greater reduction in heat production. The pigment thus appears to degrade all these forms of radiant energy into heat.

If the figures in the right-hand column of the above table carry any significance, then the function of pigment in the skin of the dark races of the tropics is (1) to degrade incident radiation and prevent its penetration, and (2) by the local rise of temperature produce a reflex diminution of heat production.

It is possible that this factor may be one of the mechanisms whereby the

therapeutic action of insolation in the treatment of tuberculosis operates. According to Rollier's belief (3), the cases which develop pigment are supposed to respond best to the solar radiations, and on the basis of the experiments of this section these are the individuals in whom heat-production would be lowered during exposure and in whom, therefore, katabolic processes are reduced and a febrile response evaded.

The mechanism whereby pigment under the influence of certain radiations lowers heat-production presents a problem requiring investigation. The reaction may possibly depend on a more intense nervous response of the heat receptors in the skin in virtue of its pigment, or it may be of an entirely different nature, namely, a photo-chemical process liberating a derivative of melanin possessing an antipyretic action comparable to that of quinine. Though the calling into play in the latter case of any mechanism of photo-sensitisation is highly improbable, especially in animals covered with thick fur, yet the possibility must be admitted. That some relation exists between the process of sensitisation and the action of pigment may be inferred from the following experiment.

*The Protective Action of Pigment against Photo-sensitisation by Hæmatoporphyrin*—In a series of interesting researches by Hausmann (4), it was shown that hæmatoporphyrin possessed the curious property of rendering animals sensitive to light. The phenomenon has also been observed in man, *e.g.*, in the experiments of Meyer-Betz (5) on himself.

The hæmatoporphyrin hydrochloride crystals for the experiments about to be described were prepared by the method of Nencki and Zaleski (6) from hæmin crystals obtained from horse's blood by their process. The neutralised fluid used for the injections yielded the spectrum (fig. 5, Plate 18) when transmitting the rays from the quartz mercury-vapour lamp.

The potency of this injecting fluid was tested on the frog's stomach by the method described in Section I. Exposure to the visible radiations only from the iron arc gave the usual response, namely, marked diminution in tone, but, on adding a milligram of hæmatoporphyrin to the 250-c c. bath of Ringer's fluid, the response was converted into a strong tonic one (fig 6).

On injecting a 20-mgm. dose into different coloured rats no ill-effect followed if the rats were kept in the dark, proving that the hæmatoporphyrin was quite innocuous in the absence of light. When, however, an unpigmented animal injected with a similar dose was subsequently exposed to any of the radiations corresponding to the absorption bands of hæmatoporphyrin, sensitisation to light was exhibited. Thus a 20-mgm. dose, followed by con-

tinuous exposure to ultra-violet light  $\lambda = 298 - 436 \mu\mu$ , killed a 40-gm. white rat in 4 hours 45 minutes and a 41-gm. white rat in 4 hours 50 minutes.

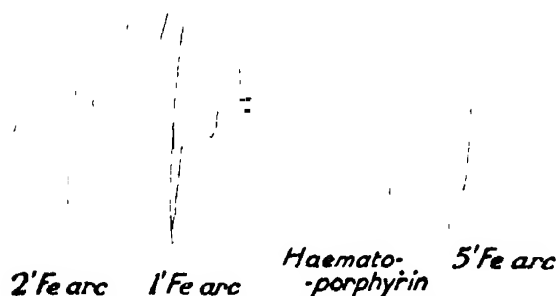


FIG. 6.—Frog's stomach: response to light before and after sensitisation.

On the other hand, a black rat of the same size given a similar dose could face any radiations with impunity; it was still alive and apparently undisturbed after 7 hours' exposure to  $\lambda = 291 - 436 \mu\mu$ , followed on the next day by a 6-hours' exposure to the naked mercury arc.

Post-mortem examination of the white rats showed no striking changes, either macroscopic or microscopic. The only indication of the cause of death was a patchy congestion and œdema in the lungs (Plate 18, fig. 7); congestion, however, could not be detected elsewhere, not even in the skin of the back, which had been in closest proximity to the radiating source. The latter was rather surprising in view of the damage produced in the skin of the hands and face in *hydroa æstivale*, a disease caused by the actinic rays of strong sunlight in subjects many of whom exhibit hæmatoporphyrin in the urine.

Since hæmatoporphyrin does not cause death in the absence of light, in the doses used, it is evident that the toxic effect is not due to hæmatoporphyrin *per se*, but rather to some derivative formed by the radiations. As the rays of wave-length  $291-436 \mu\mu$  have only a very low degree of penetrability into the tissues and certainly never reach the lungs, this derivative would appear to be formed in the most superficial layers of the dermis and to exhibit a predilection for the endothelium of the lungs. It is interesting to note in this connection that caution must be exercised in the selection of cases of pulmonary tuberculosis for treatment by heliotherapy; hæmoptysis constitutes a contra-indication.

The invulnerability of pigmented rats can only be attributed to the protective rôle played by melanin in these photo-sensitised animals. The pro-

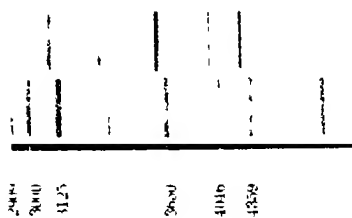


FIG. 1.—Spectrum of source of ultra-violet energy used ( $\lambda$  in Angstrom units.)

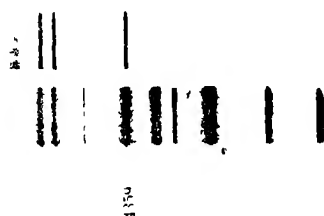


FIG. 5. - Transmission spectrum of hematoporphyrin.

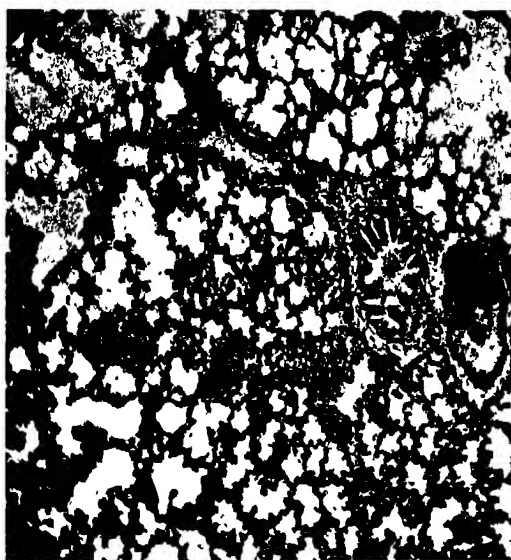


FIG. 7.—Micro-photograph of lung.



tection noted in sheep and swine affected with the disease fagopyrismus may be of a similar character : although both black and white animals are presumably photo-sensitised by phyto-porphyrin derived from the consumption of the common buck-wheat (*Fagopyrum esculentum*) the black members are completely protected (7).

The simplest view to adopt regarding the action of melanin in virtue of its position in the germinal layer of the epidermis is that the pigment intercepts the active radiations preventing them from reaching the hæmatoporphyrin circulating in the dermis ; the melanin must, of course, again emit them but in a degraded form, *e.g.*, as long heat waves which are not absorbed by hæmatoporphyrin and therefore do not sensitise it (fig. 5). A more complicated mechanism would be one involving the production of anti-body by the melanin which might either neutralise the toxic derivative produced by the light from or through the hæmatoporphyrin or antagonise its action. Since sterco-porphyrin has been shown by Garrod(8) to be a normal constituent of the urine of man and hæmatoporphyrin has been found by MacMunn (9) to occur in the skin of lower animals, our natural immunity to sunlight in the presence of hæmatoporphyrin might depend on a constant production of such an anti-body in small amount. It is usually stated, for example, that the red corpuscles are protected *in vivo* from the hæmolytic action of bile which is so easily demonstrated *in vitro*. If this is true then sufficient melanin must be present in the skin of albinos to confer this protection. Even in blondes, melaninogen granules can be demonstrated in the epidermis by the "dopa" (di-oxy-phenyl-alanine) of Bloch (10).

As a basis for the former and simpler hypothesis regarding the action of pigment measurements were made of the subdermic temperatures of white and of black rats and rabbits to determine whether the melanin in the skin and fur of these animals behaves as a purely physical agent.

*The Mode of Action of Pigment.*—In order to study the local and remote effects of pigment in the normal animal, animals were placed under the action of a hypnotic only just sufficient in amount to render them placid. For the recording of differences of temperature in the subcutaneous tissues thermocouples were used consisting of a hypodermic needle of steel carrying an insulated constantan wire which was laid bare and soldered at the point only. A pair of these was placed in series with a mirror moving coil galvanometer with a resistance about equal to that of the rest of the circuit ; the temperature could be read to a thousandth of a degree Centigrade.

In the first series of experiments a white and a black animal of the same size

and age were arranged side by side on a cotton-wool pad at equal distances from the light source. A screen was placed immediately above the backs of the animals and so arranged that equal areas of the two animals were irradiated. The thermo-couples were inserted as nearly as possible to the same depth into the loose areolar tissue in which they were easily palpable. Observing the temperature-difference at minute intervals, exposure to ultra-violet radiations, ranging between  $\lambda = 291 - 436 \mu\mu$  with a maximum at  $\lambda = 360 \mu\mu$ , revealed a greater local heating effect (fig. 8) in the pigmented animal; exposure to visible radiations produced a similar result (fig. 9, lowest curve) while the total radiations, including the infra-red, from the naked mercury arc showed the same phenomenon to a much greater degree (fig. 9, upper curves). The similarity of these curves suggests that melanin exercises a high absorptive capacity for all these different radiations.

As the site of this local heating is in close proximity to the heat receptors of the skin, the possibility of the existence of a nervous reflex counteracting the

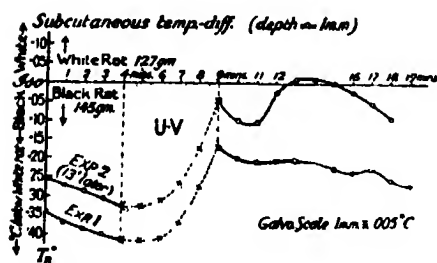


FIG. 8.

FIG. 8.—Temperature-difference between black and white rats on exposure to ultra-violet radiations.

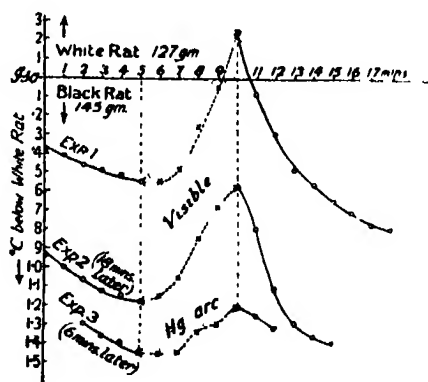


FIG. 9.

FIG. 9.—Exposure to luminous radiations and to the total radiations of the mercury-vapour lamp.

warming up of the black animal was tested by placing the thermo-couple in the side of the animal remote from the irradiated area.

A typical result is given in fig. 10, which shows that the ultra-violet and the visible radiations have no constant effect, but that the total radiations of the mercury arc induces a relatively great fall of temperature in the pigmented animal. This experiment is the more interesting because the thermo-couples were placed in the sides in contact with the cotton-wool, which practically

prevented any possibility of increased heat-loss; the effect of pigment in lowering body temperature must be a diminution in heat production, as was

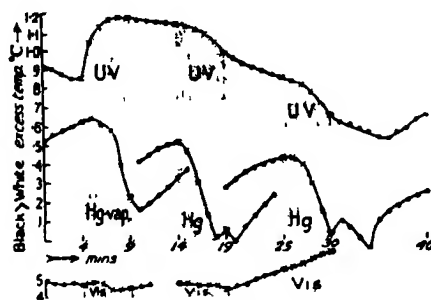


FIG. 10.—Temperature-difference of the black and white areas on the side of the animal opposite to that which was irradiated.

deduced from the results of the metabolic experiments in the first part of this section

The results of a large number of experiments exhibited a considerable degree of variation in the ultra-violet and visible responses, whereas the effects due to the total radiations from the mercury arc were uniformly constant. As a rule, the thicker the fur the less the response. In order to further reduce the variable factors, parti-coloured animals were selected with skin and fur displaying large white and black areas. One thermo-couple was placed in the subcutaneous tissue of a white patch, and the other in a black patch of the same animal. The readings corroborated the findings in the earlier experiments involving two animals, fig. 11 shows this when compared with fig. 10.

As a check on the experimental method employed above, readings were taken from two areas of an all-black rabbit (fig 12, upper set of curves) and an albino

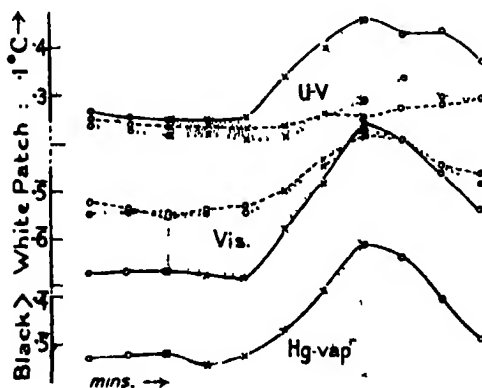


FIG. 11.—Temperature-difference of black and white patches in a parti-coloured rabbit.



rabbit (fig. 12, the three lower sets of curves). These appear, on the average, to be practically level in each case.

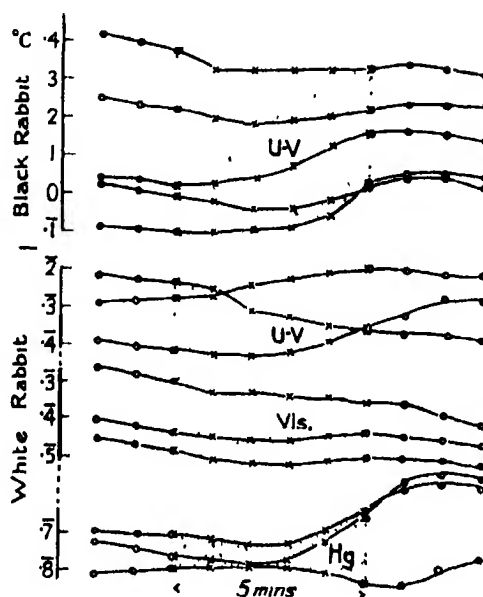


FIG. 12.—Temperature-difference in similarly pigmented areas.

Finally, the back of an albino rabbit was half-converted into a black one by depilation and painting with Indian ink. Fig. 13 shows precisely the same

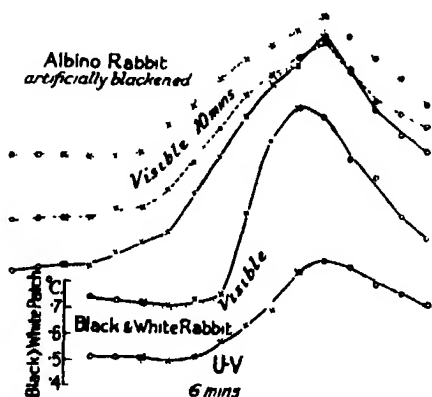


FIG. 13.—Temperature-difference of white and artificially pigmented patches.

result in this artificial case as in the earlier natural parti-coloured animals. It therefore appears that in so far as the purely physical reactions of pigment

are concerned it is immaterial whether the pigment be in the skin, on the skin, or in the fur.

The results detailed above for subdermic temperatures are in complete accord with those of Leonard Hill on surface temperatures (11).

*Conclusions.*

4. Exposure of an animal to the mixed radiations of a powerful source of light depresses its heat production to an extent which is greater in pigmented animals than in albinos for the same rise of temperature in the surrounding medium. Pigment under these circumstances appears to be a factor in diminishing heat production.

5. Thermo-electric measurements indicate that pigment, nevertheless, possesses high absorptive properties. Degradation of the absorbed radiant energy into heat produces appreciable rises of temperature in the dermis.

6. Pigment appears to protect an animal against the lethal action of certain photo-dynamic substances.

I wish to record my thanks to Mr. J. E. Barnard, F.R.S., and his assistant, Mr. Smiles, for their kind help with the quartz spectra.

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*Conduction of Excitation in the Leaf of Mimosa Spegazzinii.*

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*Introduction.*

From an investigation into the conduction of excitation in *Mimosa pudica*, carried out recently in Trinidad (17), it was concluded that normal conduction in the stem is correctly explained by Ricca's now well-known theory of conduction, based upon his remarkable and ingenious experiments on *M. Spegazzinii* (16). According to Ricca, the stimulus sets free a stimulating substance, which finds its way into the vessels of the wood, and is there carried along with the water-current. It was also concluded, however, that Ricca's theory cannot explain conduction in the leaf of *M. pudica*, for, in the leaf, Ricca's conducting mechanism is regularly masked by another more rapid mechanism, which has nothing to do with the transpiration current, and works in the phloem. That the phloem is the path of conduction in the leaf of *M. pudica* had previously been shown by Herbert (14).

There are, however, some experiments by Ricca on the leaf of *M. Spegazzinii* which he considers to show that in the leaf, as well as in the stem conduction depends on the transpiration current (16, pp. 101 and 135). Since it did not seem likely that the two species should differ in so important a respect, it seemed desirable further to investigate this question in the leaf of *M. Spegazzinii*. For the opportunity to do so I am very much indebted to the courtesy of Prof. Penzig and Dr. Ricca at Genoa, and of Prof. Buscalioni and Dr. Catalano at Palermo, who kindly provided me with all the facilities of the botanical institutes and gardens at these two places, and offered every help towards carrying out the work.

It was hoped also to study further the exact nature and path of conduction in the leaf; but, except for a few observations, this was not found possible, since the leaf of *M. Spegazzinii* was found to conduct excitation much less actively than that of *M. pudica*, and to be much less tolerant of experimental operations. A few of the experiments were performed at Genoa, in the end of October; but the greater part at Palermo, during a spell of very fine weather that lasted from the end of October to the middle of November. The plants were growing under glass; besides numerous potted plants, there was available at Palermo one large plant, rooted in the ground.

1. *Preliminary Observations.*

Only young fresh-growing leaves were found suitable for the experiments. Large mature leaves conducted excitation much less actively, though they might still respond well to direct stimulation. The following observations were made in the greenhouse on fresh young leaves attached to the plants. The lengths of petioles of such leaves were from 2 to 2.5 cm., and of each of their two pinnae from 4 to 6.5 cm. A few of the observations (at Genoa) were made at a shade temperature of 20° C., and with nearly full sunlight on the plants, but most of them (at Palermo) were made at higher shade temperatures, but in very much broken sunlight. *M. Spegazzinii* does not need such high temperatures as *M. pudica*.

1. When the tip of a pinna was cut off, numerous timings showed that excitation regularly reached the basal leaflets of the pinna after from 25 to 30 seconds. In two leaves it arrived there after only 20 seconds. The conduction times from pinna-tips to main pulvini were much more variable, and sometimes the main pulvini failed to respond at all: these times ranged from 30 to 70 seconds. For one leaf a time was recorded of only 15 seconds, but this seems to have been exceptional.

2. When leaves were cut through near the base of the petiole, excitation was conducted up to the tips of the pinnae in times from 15 to 30 seconds.

3. When a pinna-tip was stimulated by the flame of a match, conduction was much more rapid. Thus in each of six leaves so stimulated, excitation passed down the pinna and reached the main pulvinus after from 3 to 4 seconds. The times of conduction to the pinna-base were something less than this, so that the conduction rate from pinna-tip to pinna-base was regularly quite eight times greater after a burn than after a cut. This difference would be difficult to understand if the conduction depended on a stimulant travelling with the water-current in the vessels.

2. *Simultaneous Measurements of the Velocities of Excitatory Conduction and of the Water-Current in the Leaf.*

The water-current was timed, as before, by introducing strong staining solutions into the vessels, and then after a certain time rapidly cutting the leaf up into short lengths and examining it under the microscope by transverse sections in pure glycerine. As stains, 1 per cent. methylene blue or 1.3 per cent. eosin were used, the eosin being made up at 1.3 per cent. instead of 2 per cent. as previously, since less soluble at lower temperature. The 1 per

cent. methylene blue gives much the more intense stain. It was shown previously that 1 per cent. methylene blue and 2 per cent. eosin will reveal even very rapid movements of the water-current (17, p. 355); and several experiments had indicated that they can be fully relied upon to stain rapidly the vessels which they reach. The distance travelled by the stain was taken as the distance to the mid-point between the last section in which the stain was still seen, and the first section in which it failed to appear. Thus there may have been errors up to a maximum of 0.6 cm., or in four cases of 0.9 cm.

*Experiment 1. Acropetal Conduction.*—Fresh young leaves of *M. Spegazzinii* were cut off under water, and arranged with cut bases in water and blades in air, in a patch of shade before a sunny window. They were left to rest for at least two hours. Three such leaves were then gently placed with their bases in a stain (the first in 1 per cent. methylene blue, and the other two in 1.3 per cent. eosin), and at once a length of a few millimetres was cut off from their basal ends under the stain with scissors, so as to stimulate them. Excitation reached the furthest leaflets of a pinna after 40, 60 and 40 seconds in the three leaves, having travelled at rates of 8.4, 6.2 and 6.75 cm. per minute. After 1 minute 30 seconds, or 2 minutes, they were taken out and examined. The stains were found to have ascended at rates of only 2.3, 1.5 and 1.0 cm. per minute.

Another leaf was placed with its base in 1.3 per cent. eosin mixed with a little KOH to stimulate it. Excitation was conducted up at 16 cm. per minute, and reached a pinna-tip after 30 seconds. The stain ascended only at 1.2 cm. per minute

Another leaf was placed with its base in 1 per cent. methylene blue without any further stimulus. But the stain itself stimulated it, and excitation was conducted up to a pinna-tip at 8 cm. per minute, while the stain ascended at only 2 cm. per minute. It may be noticed that in this case there cannot possibly have been anything to delay the entrance of the stain into the vessels.

The above leaves were examined after only 1 minute 30 seconds, or 2 minutes, but in order to make sure that the rates of ascent of stain found in them really were the steady rates at which the water-current was ascending, two more leaves were stood in 1 per cent. methylene blue for 3 and 5 minutes respectively. After these times, the stain had ascended 2.9 cm. and 6.8 cm., at rates of 1 cm. and of 1.36 cm. per minute, about the same as those found previously. These two leaves were not stimulated by the stain.

These results show that excitation was conducted up the leaf from  $3\frac{1}{2}$  to

13 times more rapidly than the water-current was meanwhile ascending. The excitation cannot, therefore, have travelled with the water-current.

*Experiment 2. Basipetal Conduction.*—Potted plants of *M. Spegazzinii* were arranged before a sunny window, but just shaded from direct sun. The tips of pinnae of fresh young leaves of these plants were made to dip into a small dish of stain, and then immediately a short length was cut off from the tip under the stain. Care was taken that the pinna-tip was completely submerged below the surface-film all the time. The rate at which excitation was conducted down the leaf was measured, and after from 1 to 3 minutes it was cut off, rapidly cut up into short lengths, and examined microscopically. The results are given in the following table:—

Table I.

Leaf.	Examined after	Rate of Descent of Stain.	Excitation timed down to	Rate of Conduction.	Stain used.
	Min. Sec.	Cm. per min		Cm. per Min.	Per cent.
1	1 20	1	Base of pinna.	7.5	1 M.B.
2	1 30	1	" "	6	"
3	3 0	1 1	" "	11	"
4	1 30	1 8	" "	10.7	1 " eosin
5	1 30	1 4	" "	8.9	"
6	1 0	2 2	Nearly to pinna base	8	1 M.B.
7	1 0	2.9	Main pulvinus.	7.2	"

This table shows that excitation was conducted down the leaves from 2.4 to 10 times more rapidly than the stain was meanwhile descending. As before, therefore, the excitation cannot have travelled with the water-current.

An experiment similar to the last had been performed previously on *M. pudica* (17, experiment 9), but it had been found that in all the leaves except one the stain failed to enter the vessels at the cut end of the pinna. Probably this was because the pinna-tips were cut off with a knife, so that the vessels were crushed by the pressure against the bottom of the dish. This time, however, the pinna-tips were cut with scissors, and it can be seen that the stain successfully entered the vessels and was conducted down at rates about the same as those at which it had ascended in experiment 1.

### 3. Excitatory Conduction in Submerged Leaves and Leaves in Damp Air.

The leaves of *M. Spegazzinii* do not stand submergence so well as those of *M. pudica*, for often either the main pulvini or those of the leaflets lose their

power of movement, though excitation may still be conducted. But young leaves in good conditions and carefully oriented to the light will remain capable of response. The following results show the rates of conduction under water that were found in good conditions. A few experiments in which conduction either failed altogether or was extremely slow have been omitted, since it was clear that in these the conditions must have been somehow unfavourable.

Shoots of *M. Spegazzini* were cut under water and kept submerged in bowls of water in a horizontal position, and pointing towards the light, so that it struck the upper surfaces of the main pulvini. The leaves soon opened again. After some hours they were stimulated by being cut, still under water, with scissors, care being taken not to shake or drag them. The following results were obtained :—

*Experiment 3*—Genoa, 25 10.24. The submerged shoots were exposed to nearly full sunlight.

1. *Basipetal Conduction*.—The tips of pinnæ of four leaves were cut off, and excitation reached the main pulvini after 10, 10, 7, and 45 seconds.

In two other leaves from which pinna-tips were cut off, excitation reached the basal leaflets after 5 and 10 seconds. The above leaves were in water at 23° to 26° C. and had been submerged for from two to three and a half hours.

2. *Acropetal Conduction*.—Two leaves were cut through in the petiole, being meanwhile held stationary with forceps. Excitation reached the tips of the pinnæ after 10 and 2 seconds. These leaves were in water of 23° and 26°, and had been submerged for two hours and for three and a half hours.

In some other leaves, however, when the petioles were cut, the leaflets were seen to close instantaneously. They had probably been directly stimulated by some mechanical shock or jolt caused by the cut, though the point needs to be further investigated. But in the above two leaves it was noted that the leaflets closed in regular sequence up the pinnæ, so that excitation must have been conducted up them in the times recorded.

*Experiment 4*.—Later, at Palermo, shoots were submerged in the greenhouse, as before, except that they now received only very much broken sunlight.

1. *Acropetal Conduction*.—The leaves of three submerged shoots were cut through in the petioles, and excitation passed up to the tips of the pinnæ in 10, 12 and 5 seconds. The first of these had been submerged for 3½ hours, the second overnight, the third for 23 hours.

2. *Basipetal Conduction*.—A pinna-tip was cut off from the leaf of a shoot submerged for 23 hours, and excitation was found to reach the basal leaflets

after 10 seconds. Two detached leaves also, previously cut off under water at the base of the petiole, were stimulated by cutting off pinna-tips, and excitation was conducted down to the basal leaflets in 5 and in  $1\frac{1}{2}$  seconds. They had been submerged for two and for three and a half hours. Often, however, such detached leaves were found to lose their conducting power.

If these conduction times are compared with those recorded in section 1, for leaves in air, attached to the plants, when similarly stimulated by cuts, it will be seen that the velocities of conduction under water are very much the greater.

It having been pointed out to me by Dr Ricca that the transpiration current may not be entirely suppressed in submerged shoots, the rates of ascent of staining solutions in their stems were measured as follows —

*Experiment 5* Three shoots were cut under water and similarly submerged in bowls before a sunny window covered with a screen of close-meshed muslin. This was considered to allow them about the same intensity of sunlight as was received by the shoots of experiment 4. After 1, 2, and  $2\frac{1}{4}$  hours respectively, their basal ends were gently lifted just out of the water, and inserted into a narrow tube, open at both ends, containing 0.75 per cent. methylene blue. They were held there for five minutes, and then examined as before. Care was taken that the cut ends were not obstructed by bubbles of air.

The stain was found to have ascended at rates of approximately 0.8, 1.3 and 0.8 cm. per minute.

It must be mentioned that in the second shoot the terminal leaflets of one or two pinnae were just floating on the surface, and that in the third shoot they had been so doing until the last half-hour.

*Experiment 6* —For comparison, two shoots were cut off under water and set up with bases in water and leaves in air, in a light similar to that of experiment 5. After  $2\frac{1}{4}$  hours' rest, one was transferred to 0.75 per cent. methylene blue for 1 minute 30 seconds and then examined. The stain had ascended at approximately 5.4 cm. per minute.

The other was similarly treated, except that when it was dipped into the stain, a short length was cut off, under the stain, from its base. It was examined after 2 minutes, and the stain was found to have ascended at approximately 5.6 cm. per minute.

These results show that in the shoots in air, in conditions otherwise similar, the water-current was ascending from four to seven times more rapidly than in the submerged shoots, although these had not been submerged for so long



as the shoots of experiments 3 and 4. Since, therefore, by submerging shoots under water, the velocity of the excitatory conduction in the leaves set up by a cut is greatly increased, while the velocity of the water-current is many times diminished, it is clear that in the leaf excitation is not conducted with the transpiration current

By comparing these results with those of experiment 1, it can be seen also that the transpiration current must ascend considerably more rapidly in the stem than in the leaf, though some allowance must be made for the much-reduced sunlight falling on the shoots of experiment 6.

It seemed further of interest to determine whether very damp conditions would increase the rate of conduction in the leaf to a less extent. Accordingly, after various preliminary trials, the following experiment was arranged —

*Experiment 7.*—Several "stem and leaf" preparations were arranged in a glass bowl with stem portions in water and leaf-blades floating on the surface of the water, over which the air was kept damp by a sheet of glass covering the bowl. Other similar preparations were arranged, for comparison, with stem portions in water and leaf-blades in normal air. Both lots were placed together in much broken sunlight in the greenhouse. After a rest of some hours, they were stimulated by cutting off a pinna-tip. The results are given in the following table —

Table II — Conduction Times in Damp and Dry Air

Date	In Damp Air			In Dry Air			Previous Period of Rest
	Number of Leaf.	To Base of Pinna	To Tip of Other Pinna	Number of Leaf.	To Base of Pinna.	To Tip of Other Pinna	
1.11 24.	{ 1	Seconds	-	6	Seconds	Seconds	Overnight
	{ 2	10	-	7	40	100	
	{ 3	15	-	8	30	60	3 hours approx
31 10 24	{ 4	25	-	9	55	72	
	{ 5	20	-	—	35	45	
		25	-		—	—	

The above table shows that excitation was conducted more rapidly down the pinnae in damp air than in dry air. It was conducted more rapidly also than in similar leaves attached to the plant, but less rapidly than in leaves of submerged shoots. Thus with increasingly damp conditions, the velocity of the excitatory conduction in the leaf set up by a cut is progressively increased. This may not, however, be true for the much more rapid conduction set up

by a burn on a pinna-tip, which seemed even in dry air to reach nearly its greatest possible velocity.

The table shows also the curious fact that in damp air the excitation did not cross over from the stimulated pinna to the other pinna. It did not cross over in submerged leaves either. But in dry air, although excitation travelled more slowly down the stimulated pinna, it yet crossed over into the other and was conducted up to its tip. Yet in the leaves in damp air also it could be rapidly conducted acropetally, for when the first two leaves of the above table were cut through in the petiole, excitation was conducted up the other pinna and reached nearly to the tip after times of about 8 and 12 seconds. A similar result was often obtained in submerged leaves. It must therefore be just the actual crossing over from one pinna to the other which in damp conditions is for some reason prevented.

#### *1. Conduction with Acceleration*

It has been pointed out by Borzi and Catalano (3, p. 7) that in the leaf excitation is conducted with increasing speed as it progresses. This observation was made for basipetal conduction only, and they consider that the explanation is that the lower part of the pinna is so constructed as to conduct better than the upper part. But by timing conduction in both directions it could be determined whether the process of conduction has not in itself also a tendency to accelerate.

In making such timings it must be remembered that there is an additional period of delay between stimulation and response of the leaflets, which is made up of the latent period of their pulvini and the time taken by the excitation to pass out to them from the pinna-rachis. This delay was allowed for in the following way :—

*Experiment 8.*—The tips of pinnae of young leaves were cut off just behind the terminal pair of leaflets. As a result, a few of the pairs of leaflets nearest to the tip (usually two or three) were found to close instantaneously, having probably been directly stimulated by the mechanical strain or jolt. The remaining length of pinna, in which the leaflets had not closed, was now rapidly measured. After several seconds the first pair of leaflets of this remaining length was seen to close, excitation having reached it; and from this moment as a starting-point a note was made of the time taken by the excitation to travel first to a point half-way down the remaining length of pinna and then to the basal leaflets. With this method, the period of delay makes no difference, since it would have to be subtracted from all the recorded times alike. The

timings were made on young leaves, attached to the plant, in much broken sunlight. The results are given in the following table :

Table III.—Basipetal Conduction.

Number of Leaf.	Conduction Time over First Half of remaining Length of Pinna	Conduction Time over Second Half Length.
	Seconds	Seconds
1	20	7
2	17	6
3	25	10
4	17	6
5	18	6
6	30	22
7	33	8
8	31	21

This table shows that basipetal conduction was always more rapid in the second half of the measured length, and that in five of the eight leaves it was nearly three times or over three times more rapid. Yet if excitation were conducted down the leaf with the water drawn back down the vessels, it would be expected that it would be conducted most rapidly at first, while the tension on the water columns was greatest.

Acceleration was sometimes found in acropetal conduction also, when the stimulus was of just the right strength, as follows :—

*Experiment 9*—Timings were made on fresh and active young leaves attached to the plants in broken sunlight. One leaflet of the basal pair of leaflets of a pinna was cut through. The moment at which the next pair responded was taken as the starting-point, and from this moment a note was made of the times taken by the excitation to travel first up to a point half-way along the remaining length of pinna, and then up to the end. In this remaining length, however, the extreme tip carrying the terminal pair of leaflets was not included, since these leaflets differ from the others and often delay their response. The results are given in Table IV.

From this table it can be seen that in four leaves out of six conduction was nearly equally rapid in the two halves of the measured length, while in the other two leaves it was rather more rapid in the second half.

The acceleration is therefore very much less in acropetal than in basipetal conduction. Provided, then, that the nature of conduction is the same in both directions, it follows that the lower half of the pinna does indeed conduct more actively than the upper half. But this cannot be the only

Table IV.—Acropetal Conduction

Number of Leaf.	Conduction Time over First Half of Remaining Length	Conduction Time over Second Half
	Seconds	Seconds
1	30	30
2	18	19
3	32	31
4	20	12
5	16	17
6	30	25

factor causing the great acceleration in basipetal conduction, for if it were, one would expect a corresponding acropetal deceleration. There must, therefore, with the above condition, be a tendency towards acceleration which depends on the nature of the conducting process itself. The two factors acting together cause the great basipetal acceleration while in acropetal conduction they nearly counteract one another.

It is not suggested that an acceleration would be found in all circumstances. For if the physiological conditions are less favourable, or the stimulus is too weak, conduction begins to slow down and die out before the end of the pinna is reached, while if the stimulus is too strong, rapid conduction follows so quickly that an acceleration cannot be detected by the eye.

### 5 Multiple Conduction

When a leaf of *M. Spegazzinii* is stimulated by a cut in the petiole, it is often found that the leaflets do not close together completely when the excitation reaches them but rise up through some lesser angle only. After some seconds, a second wave of excitation passes up the pinna, making the leaflets rise a little higher, and sometimes several such waves pass up before the leaflets finally meet together. This curious phenomenon was mentioned to me by Dr. Ricca, who had, however, his own interpretation of it: it will be called "multiple conduction." If the first wave does not reach the tip of the pinna, the next wave usually travels farther. The interval between two waves may be quite long, as was found in one leaf in which the first wave passed half-way up one pinna after 15 seconds, and then nothing more happened until after 1 minute 25 seconds, when a second wave passed up the pinna nearly to the tip.

The successive excitations must originate in the conducting tissue of the petiole or pinna-rachis, and not in the pulvini of the leaflets, since otherwise the successive pairs of leaflets could hardly keep time so perfectly as to give the

appearance of a wave of excitation passing up the pinna. This suggests the question whether in the multiple response of the main pulvinus of *M. pudica* (1, p. 77) the successive excitations originate in the motor cortex or in the conducting tissue.

It was only acropetal conduction that was ever observed to be multiple. But it is possible that sometimes basipetal conduction also may be multiple, though it could not usually reveal itself as such by waves of partial closure; for in basipetal conduction, even when it is very slow, the leaflets regularly close completely the first time.

A phenomenon nearly similar to the above was observed by Haberlandt in the leaf of *Biophytum sensitivum* (12, p. 35).

#### 6. Leaf Conduction and the Question of the Tube-Cells.

The results reported above show that in the leaf of *M. Spegazzini* excitation is regularly conducted by some mechanism that has nothing to do with the water-current in the vessels. The same conclusion was reached previously for the leaf of *M. pudica*, in which the main path of this conducting mechanism is the phloem, as had been shown experimentally by Herbert (14), and as was confirmed (17, p. 363). This result agrees also with the result of a single experiment on the leaf of *M. Spegazzini* by Buscalioni and Muscatello (7, p. 15). It can hardly be doubted, therefore, that in the leaf of *M. Spegazzini* also the phloem regularly conducts excitation, though unfortunately *M. Spegazzini* withstands operations so much less well that it would be extremely difficult to prove this by similar experiments. Certain criticisms of these conclusions (8) have been answered elsewhere (18).

It remains to be considered what may be the nature of conduction in the leaf. Haberlandt has brought forward the theory that excitation is conducted by changes of pressure in the "tube-cells" (11). Since it has been concluded, in agreement with Ricca, that in the stem excitation is normally conducted in the wood, and not in the phloem at all, the question of the tube-cells need here be considered only for the leaf.

Haberlandt's theory has attracted much attention, though not supported by any direct experimental evidence. Further, his suggestion that excitation is conducted by pressure-waves similar to those in elastic-walled tubes (13, p. 644) is impossible when the stimulus is a cut. For, as he himself has admitted (11, p. 61), such waves can only be transmitted when the cut end of the tube is immediately closed again, whereas the tube-cell sap continues to flow out for several seconds at least. Moreover, the formula for the velocity of these waves

shows that it is independent of the strength of the stimulus (9), whereas in the leaf of *Mimosa* the velocity does depend on the stimulus (section 1).

Several of the experiments reported in this paper or previously are unfavourable to Haberlandt's theory. Thus it was repeatedly found that if the stem of *M. pudica* was cut into deeply enough to cause the drop of tube-cell sap to exude, but not so far as the cambial region, no excitatory conduction followed (17, p. 353, cf also p. 370). In *M. Spegazzinii* also the stems of plants in pots were cautiously cut into directly below a fresh and sensitive young leaf and only a short distance from it, just until the tube-cell sap was seen to exude, again the leaves did not respond. Now Haberlandt, having cut through a petiole of *M. pudica*, measured the amount of sap that issued from the cut end of the part still attached to the plant, and calculated that the tube-cell sap must have been displaced for a distance of at least 24.2 cm. down the stem (11, p. 40). It will therefore hardly be doubted that correspondingly when the tube-cell sap was caused to exude by a cut in the stem, the sap was thereby displaced in the tube-cells of the leaf above. Since, therefore, the leaflets did not close, it follows that a displacement of the tube-cell sap in the leaf did not produce the effects of a conduction of excitation.

Again, it has been pointed out by Borzi and Catalano (3, p. 11-12) that excitation can be conducted by parts of *Mimosa* that contain no tube-cells, namely, by the lateral roots, the cotyledons, and the tips of the leaflets. The roots may very probably conduct by Ricca's mechanism, for the rate of conduction in them is stated by Borzi (2) to have been only from 15 to 20 cm. in 3 to 5 minutes. But the leaflet-tips and cotyledons of *M. Spegazzinii* still conducted excitation, as I found, after having been totally submerged for 2 hours, when it was unlikely that they could be conducting by Ricca's mechanism. In the submerged seedlings, when the tip of one cotyledon was cut off, excitation was conducted to the pulvulus of the other in 1½ or 2 minutes.

The fact, too, that in the leaf excitation is often conducted with acceleration (section 4) suggests some relay mechanism, in which energy is released at successive stages along the conducting path, rather than a simple physical wave. It seems, therefore, for many reasons extremely unlikely that it is by changes of pressure in the tube-cells that excitation in the leaf is regularly conducted.

But if this is so, there is then no reason for supposing that the tube-cells, which contain only scanty protoplasm, are the regular conducting elements at all. For several ingenious experiments by Bose show that conduction in

the leaf is in all probability a true physiological process, and that in some ways it resembles conduction in animal nerves (5, p. 161 *seq.*, and 6, p. 115 *seq.*). (The conflicting results of Fitting (10, p. 507), who states that acropetal conduction in the petiole of *M. pudica* was "not made at all slower" by cooling a zone to 0°-2° C., needs to be reported more in detail.)

It therefore seems more likely that excitation in the leaf is conducted by the "cambiform" cells, as suggested by Borzi and Catalano (3, p. 12). For these, as they point out, contain plentiful protoplasm and conspicuous nuclei, and are the only non-lignified cells of the vascular bundles that are present in all the parts that have been shown to conduct excitation. Since they are found not only in the phloem but also on the inner side of the xylem in the petiole and in between the vessels in the leaflets, the physiological process of conduction may not be entirely confined to the phloem.

It may be noted that excitation can be rapidly conducted in the leaves of "sensitive" plants of other genera that have no tube-cells (3, p. 12), and also in leaves of plants that do not respond by movements at all. In the latter, the excitatory conduction can be revealed by the electric change that follows the stimulation of a distant point, as shown by Montemartini (15), and Bose (1, pp. 21 and 452). It seems therefore, that the chief peculiarity of *Mimosa* is, after all, not that it conducts excitation, but that it reveals the conducted excitation by conspicuous movements (*cf* Bose, 4, p. 21 *seq.*). Since, therefore, it is not necessary to consider the whole mechanism of conduction and response in *Mimosa* as something specially evolved as a single function, there seems no reason why it should be thought surprising that there should be more than one kind of conducted excitation that can excite the sensitive tissue of the leaf, and so bring about response.

### *Summary*

1. Simultaneous determinations were made of the velocities of the water-current and of the excitatory conduction set up by cuts in leaves of *M. Spegazzinii*. In both directions the latter was found to be many times the more rapid. The excitatory conduction set up by a burn is much more rapid again.

2. The excitation set up by a cut is conducted in both directions much more rapidly in the leaves of shoots that have been totally submerged for several hours than in leaves in air attached to the plants. Yet in the submerged shoots, the velocity of the water-current is greatly diminished.

3. After a stimulus of the right strength, excitation is conducted down the pinnæ of *M. Spegazzinii* with great acceleration. It appears that this

acceleration must depend in part on the nature of the process of conduction itself.

4. The phenomenon of "multiple conduction" is described.

5. It is concluded that in the leaf of *M. Spegazzinii* excitation is regularly conducted by some mechanism that has nothing to do with the water-current

6. From various experiments and considerations it is concluded that excitatory conduction does not depend on changes of pressure in the tubercells, even in the leaf.

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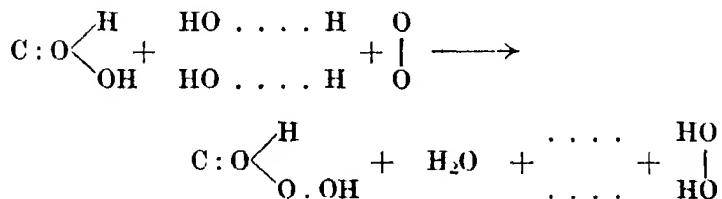
*Studies on Enzyme Action. XXIV.—The Oxidase Effect and the Phenomena of Oxidation in General: Carbonic Oxide.*

By HENRY E. ARMSTRONG, F.R.S.

(Received March 2, 1925)

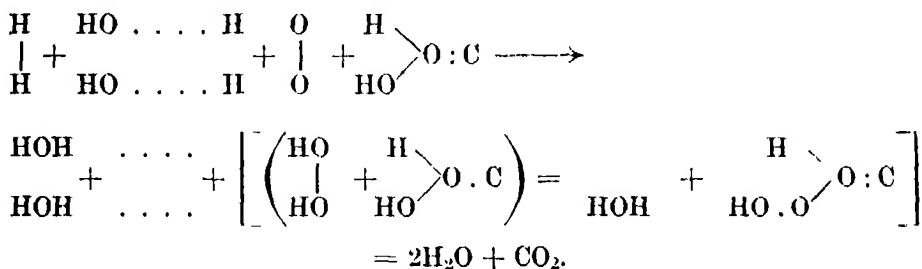
In 1904 I presented a brief communication to the Society on "The Retardation of Combustion by Oxygen" ('Proceedings,' vol. 74, p. 86). Friends smiled at the title and no notice has been taken of my argument. Of late years, however, Moureu and his fellow-workers have brought before the French Academy a series of masterly studies of the inhibition of the oxidation of highly oxidisable materials, such as acrolein and linseed oil, by substances which are themselves oxidisable, including phenol and potassium iodide. The explanation they have put forward, involving the recognition of an active antagonism between peroxides, which constitute a reversing mechanism, is essentially that I have long held, the difference being mainly that I have gone more fully into the details of the process. In a recent fascinating brief communication to the Chemical Society ('Chem. Soc. Journ.,' 1925, p. 1), Moureu and Dufraisse discuss the stoppage, by carbonic oxide, of the interaction of hydrogen and oxygen, at a platinum surface, contending that this also is an instance of inhibition owing to the antagonism of incompatible "peroxides," formed in the initial stages of change. I fully share their views and regard this communication as the settlement of a problem of prime importance which has been open to solution since early in last century (1833).

I have discussed the peculiar "indifferent" behaviour of carbonic oxide on many occasions before the Society and elsewhere. I would now take one further and perhaps final step, and proclaim it to be *per se* an incombustible gas. If the process of combustion be, as I have frequently argued, one in which an electrolytic determinant is concerned (comp. First Messel Memorial Lecture, 'Soc. Chem. Ind.,' 1922, pp. 253-270r), primarily as represented by the schematic equation



it is one involving the electrolysis of water, and the "energy" developed in the interaction must be at least equal to that involved in the combustion of hydrogen. Actually, the heat of combustion of carbonic oxide is below that of hydrogen (67960 : 68360).

Therefore, the change should not take place. An explanation may be found in the assumption that when a moist carbonic oxide mixture is sparked or fired, some hydrone is decomposed and sufficient hydrogen set free to act in a "depolarising" circuit together with the oxide, as thus :—



The observations on carbonic oxide made by Bone and his colleagues seem to me all to meet with a natural interpretation when considered from the point of view now advocated.

*The Oxidase Effect.*—Having dealt with hydrolytic enzymes, in a long series of communications to the Society, of late years, with its aid I have turned my attention to the even more mysterious class of intermediary agents, the *Oxidases*—using this term in its widest sense but excluding soluble "peroxides." The *Oxidases* are commonly regarded and usually spoken of as *Enzymes*.

The question to consider is, What is an Enzyme? I would say, first, a catalyst, a solid particulate agent, which has the faculty of attracting to and assembling upon its surface the substances whose interaction it promotes.

Second, a *strictly selective* catalyst—one that, in some way, corresponds in structure with the affected substance; in other words, one that fits upon it through absolute likeness, not merely as lock and key, as Emil Fischer suggested. Enzymes are so selective that, apparently, they act only upon like, never upon analogous, compounds. Thus *invertase* will hydrolyse both cane-sugar and raffinose—but raffinose is but cane-sugar with a galactose-glucose tail. The glucosides (a large class) all contain one glucose.

An Enzyme being thus defined, the *Oxidases* do not fall under the definition; they are but catalysts, which promote the oxidation of allied substances but not of like substances alone. *Tyrosinase*, for example, will determine not only the oxidation of Tyrosin (to Melanin) but also of a number of phenols.

The only instances of apparently selective oxidation, presumably by Oxidases, are those afforded by *Bacterium aceti* and *B. xylinum* and by *Xanthin oxydase*, which last affects both Xanthin and Hypoxanthin but none of the allied compounds.

*Bacterium aceti* is entirely peculiar in that it promotes the oxidation of ethylic and propylic alcohols but not of methylic and isopropylic. In absence of all precise knowledge of the process, it can only be supposed that either peculiarities in configuration of the molecules or energy differences—perhaps both—are at the bottom of such discriminative attack; may be antagonistic changes are also to be reckoned with. Until we can learn to think in the solid, it will be difficult to master such problems.

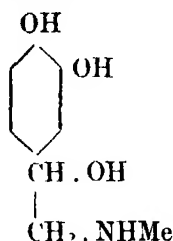
Meanwhile, I am attempting to deal with the problem from the synthetic side. It is clear that the phenomena of oxidation in the living cell, which are among the most important of vital phenomena, are at present but superficially understood and superficially interpreted. When a student, nothing struck me so much as the production of molecular oxygen from atomic oxygen by the interaction of oxides—e.g., hydrogen peroxide and silver oxide, hydrogen peroxide and permanganate. The intense affinity of oxygen for oxygen, thus made obvious, has always been before my eyes. To-day, no one thinks in terms of oxygen—only the hydrogen ion is considered: we fail to see that the Giver-of-Life is also the Giver-of-Acidity and, through water, the connecting link in the vast majority of chemical changes.

#### *Addendum. March 12, 1925*

*Vital Oxidation Phenomena.*—The extent to which the inhibition of oxidation is of vital significance is yet to be appreciated. Moureu and Dufraisse have, indeed, pointed out the possible bearings of their work and have dealt very generally with the problem: Moureu has even suggested that thyroxin may act by controlling oxidation. I have long believed and taught that hydrogen cyanide kills because it staves oxidation at nerve centres. At present, we have no explanation of the manner in which the functions of the various secretions of endocrine organs are exercised, and even more mysterious are the adjuvants in food, which now figure so largely in all discussions on nutrition. It has long seemed to me important to attempt, in the first place, to explain the function of a substance of known origin and localised activity, and of established chemical structure, such as Adrenaline; if we cannot account for the special behaviour of so simple a substance, we are not likely to be able to deal with agents of more complex character.

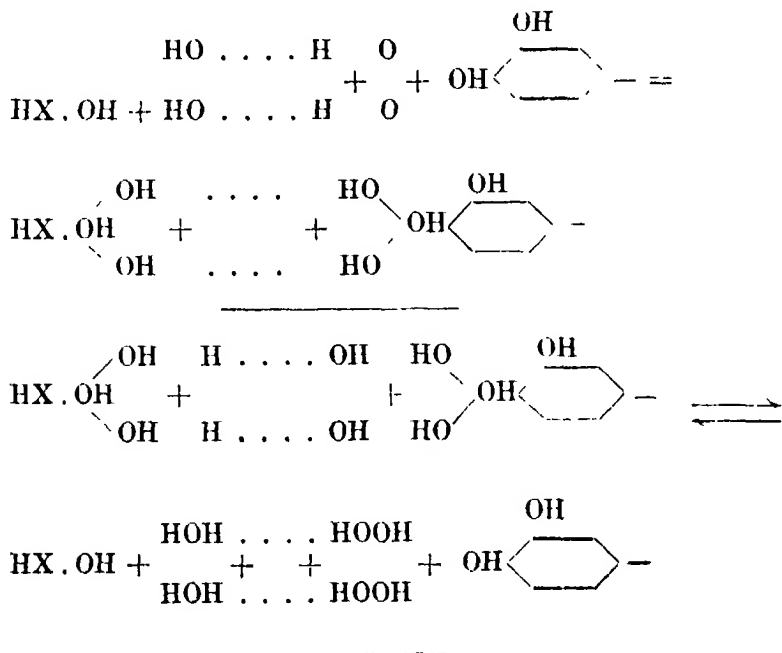
Adrenaline is remarkable in that it causes constriction of the blood vessels, even when used in very high dilutions, its efficiency being far above that of any other substance producing a like effect, including its optical opposite,  $\delta$ -adrenaline. It is a product of the sympathetic system and it acts especially upon the sympathetic system. We may well suppose that it is specially attracted to sympathetic centres in virtue of the structure of its asymmetric centre, if not of the molecule as a whole.

Adrenaline is a derivative of Pyrocatechol of the formula



Pyrocatechol is one of the substances that Moureu and Dufrasse have found to be most active in antagonising oxidation.

The possible action of Adrenaline may be pictured as follows:—



The superior activity it exhibits may be referred to the fact that, by fitting

the sympathetic centre, enzyme-like, it specially controls oxidation at the centres. This explanation may at least serve to direct further inquiry, not only with adrenaline. I would venture to suggest that it may be worth while to consider whether phloridzin-diabetes, always a matter of wonder to me since I became aware of the peculiar action of the glucoside, may not be the consequence of inhibited oxidation.

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*An Explanation of the so-called Intertraction Phenomenon between Solutions, and the Molecular Significance of Negative Surface Tension.*

By N. K. ADAM, M A , Royal Society Sorby Research Fellow, and  
G. JESSOP, Ph D.

(Communicated by Sir William Hardy, Sec.R.S.—Received March 7, 1925.)

(PLATES 19-21.)

Sir A. Wright\* and Schoneboom† have observed that when certain solutions‡ are superposed on other solutions, mixing occurs not by simple diffusion but by the development of streamers or "pseudopodia," which start from the interface and make their way upwards and downwards through the body of the two solutions. The phenomenon is attributed to a special force, "intertraction," which aids the mixing; and Schoneboom suggests that this is a spontaneous extension of the interface between the solutions, under the influence of the peculiar capillary forces present in this region.

Such a force at the interface would be of the greatest importance for the theory of capillarity. But in studying this phenomenon we have observed facts which are wholly out of accord with such an explanation. It seems that surface tension has nothing to do with the phenomenon, and that the two

\* 'Roy. Soc. Proc.,' B, vol. 92, p. 118 (1921).

† 'Roy. Soc. Proc.,' A, vol. 101, p. 531 (1922).

‡ Schoneboom states that intertraction is obtained "with *all* inorganic and organic compounds without any exception, provided . . . that the substances are sufficiently soluble in water, and that there is not too great a difference in specific gravity between the upper and lower solutions." The sequel shows that this is not the case, a definite difference in diffusivity between the dissolved substances in the upper and lower solutions being necessary for the phenomenon; it is also essential that the slower diffusing solution should be on top.

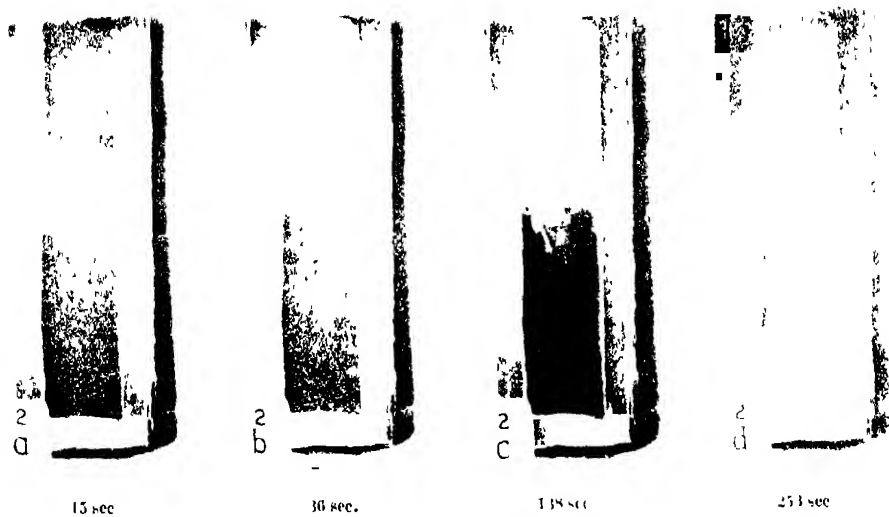


FIG. 2. Salt, 1 0365, albumen, 1 034

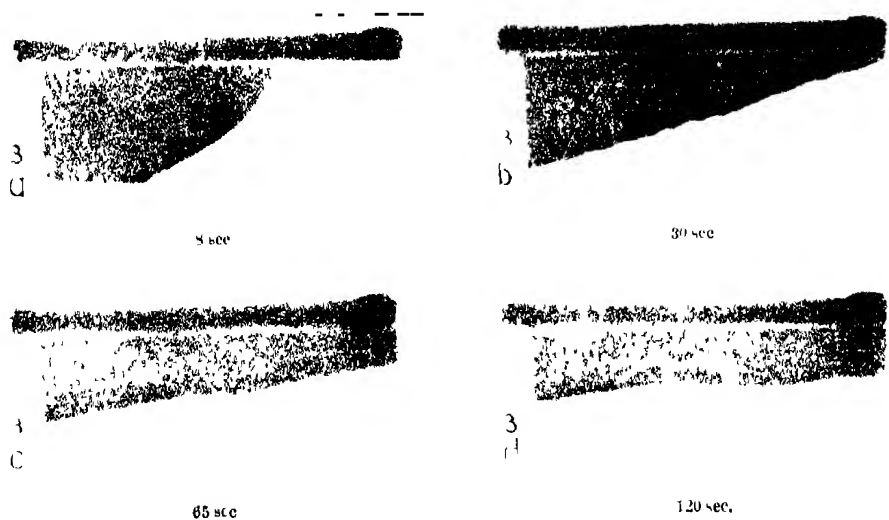
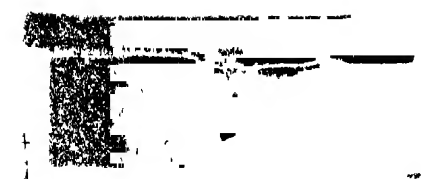


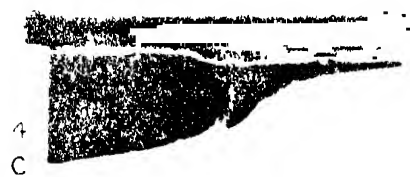
FIG. 3. Salt, 1 032, albumen, 1 034.



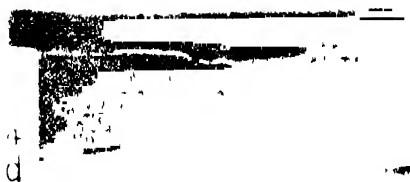
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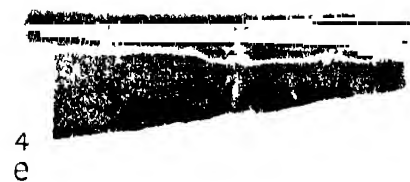
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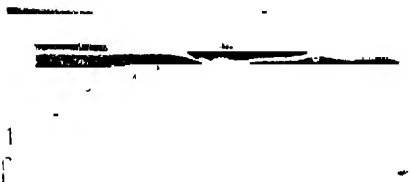
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30 min

FIG 4.—Salt, 1 034; albumen, 1 034

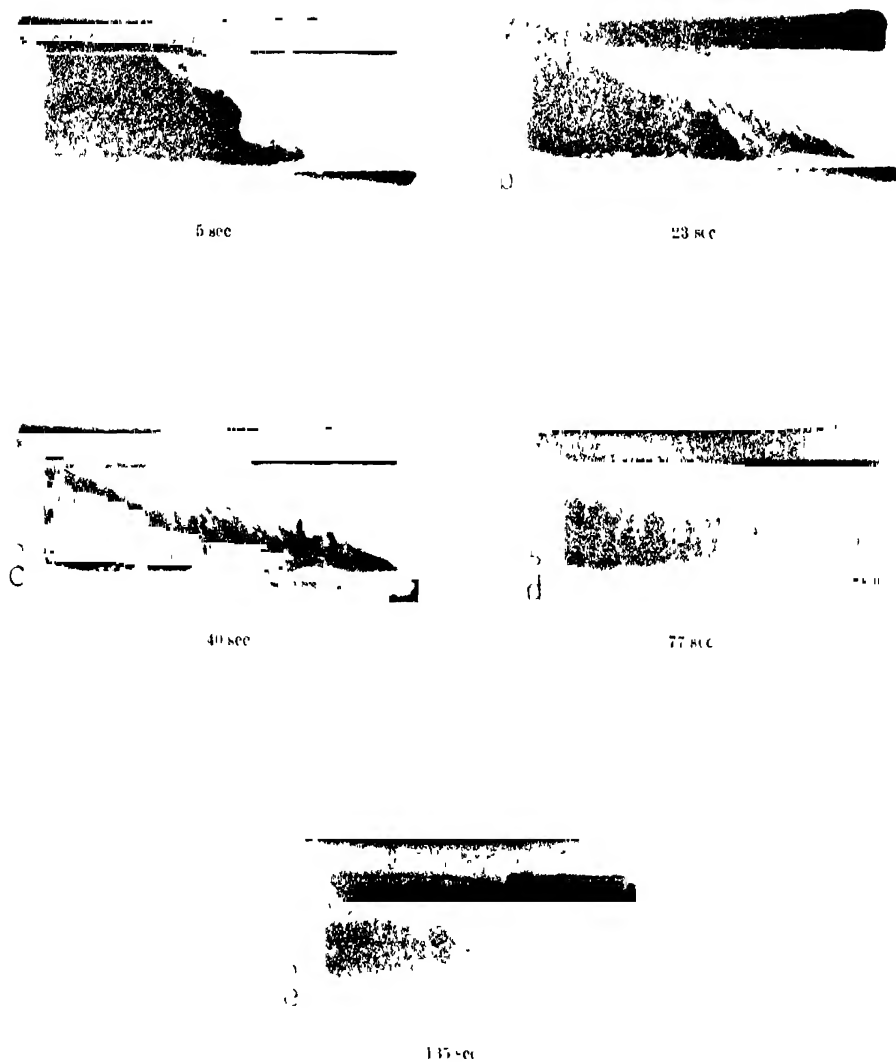


FIG. 5—Salt, 1 0365, albumen, 1 034.





essential factors are the difference in the rates of diffusion of the solutes in the two solutions, and the direction of gravity. Moreover two rather different phenomena are exhibited in different circumstances.

We think the correct explanations are as follows. Suppose two solutions of solutes A and B, in the same solvent, A diffusing faster than B, are placed one above another carefully.

*Case 1.*—Suppose first the density of solution B is the less, and that the difference in density is not great. At the instant of contact of the solutions, there is a plane surface of separation if the solutions have been sufficiently carefully placed in position. This is, of course, unattainable in practice, and there will always be irregularities in the surface of separation. From the moment of contact on, A diffuses upwards into B and B downwards into A. But since A diffuses faster than B, in a short time the layer of B just above the interface will have gained more solute than it has lost, and its density will be increased. If the difference in rate of diffusion is great enough, and the initial difference in density not too great, these layers of B will in time become denser than A. Thus the hydrostatic equilibrium of the liquids will be upset, and B will descend into A. The form of the motion in this case is determined as follows. At a point where there is initially a slight bulge of B down into A, conditions are specially favourable for rapid diffusion, as the interface is particularly large relatively to the volume of fluid B just behind. Therefore the increase of density of B will be more rapid in a bulge downwards than elsewhere, and the bulges will tend to be accentuated and to develop into streamers which will descend to the bottom of the vessel.

In a similar way, streamers of A will develop and move up into B, for at any point where there is a slight bulge of A upwards into B, the loss of salt from A to B, and the decrease of density of A, are more rapid than elsewhere. Since as a rule the initial bulges upwards and downwards will occur alternately, streamers will usually start upwards and downwards alternately from the interface.

Naturally much commotion is caused in the liquid by these streamers, and this aids mixing. The intertraction is, however, essentially an inversion of the layers of liquid, and the mixing is only secondary. If B is coloured and A uncoloured, usually after a few minutes the motion ceases, and the vessel is nearly uniformly coloured; but often, and especially when the streaming has been rapid, the lowest layers are deeper in colour than the remainder. In one case, using a hydrochloric acid solution coloured blue (A), and magnesium sulphate (B), A being 0.007 denser than B initially, very violent streaming

occurred in a few seconds, after two minutes the motion had ceased, and the upper half of the vessel was deep blue, the lower half being only faintly coloured. In this case the difference in diffusion coefficients was about 2.0 sq. cm. per day, the largest difference used, and the difference in density was small.

If the difference in density is too great, the streamers do not develop. In aqueous solutions, when the difference in coefficients of diffusion was about 0.3 sq. cm. per day, well marked "intertraction" streamers were observed if the difference in density did not exceed 0.03, but if it exceeded 0.05 there was no intertraction. Most of our experiments were done in narrow glass cells, 7 cm. long by 2 cm. deep by  $1\frac{1}{2}$  mm. thick, open at the top edge. In these narrow cells we frequently noticed that the greater the difference of density between the solutions, the thinner were the streamers. The limits of difference of density within which the streamers develop have not been worked out, but probably a greater difference of density is permissible with greater differences of diffusion coefficient than with smaller.

*Case 2*—A and B placed in contact, side by side, in a shallow pool on a glass plate. If the pool is only about  $\frac{1}{2}$  mm. deep, there is no rapid movement of the liquids into one another, except for a disturbance at the moment of first contact, which may result in a curious stationary pattern being formed for a few minutes. No streamers develop from one liquid to the other. We have tried this with albumen (B) and sodium chloride (A), and with hydrochloric acid (A) and potassium nitrate (B), two pairs of substances which give very marked streaming under the conditions of Case 1. Except for the direction of gravity all forces between the liquids are the same as in Case 1. This proves that capillary forces are not concerned in the phenomenon.

*Case 3*.—B denser than A, liquids superposed. We have never observed streamers like those of Case 1, when the rapidly diffusing solution is above the other. If the difference of density was more than about 0.025, with aqueous solutions no motion of the boundary was observed. If the difference was less than this, local disturbances of the boundary often occurred, which were always few in number, and quite different from the streamers of Case 1. They began as a slight elevation of the lower liquid at the boundary, which assumed a sharp point and sent up a very thin wisp of the lower solution into the upper; there was a rapid circulatory motion of the liquid visible at these points. With aqueous solutions these vortices were usually small and often moved slowly along the boundary; but with alcoholic solutions they were more marked and more persistent. Occasionally, one vortex would disappear and another appear at another point. Fig. 1 is a sketch of the vortices obtained

with alcoholic solutions of resorcin (B) 0·832 s.g., and iodobenzene (A) 0·826 s.g., the difference in diffusion coefficients being about 0·45.

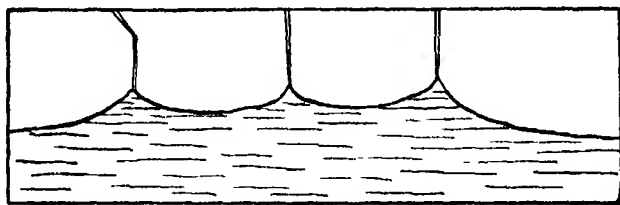


FIG. 1.—Vortices with rapidly diffusing solutions uppermost

Probably the explanation is that the diffusion of the solutes across the boundaries causes the upper layers of B (the lower liquid) to become denser, and the lower layers of A to become lighter. There is, therefore, set up a circulation in each liquid, through the lower layers changing place with the upper. This produces eddies in certain places, and if the difference in specific gravity of A and B is not too great, the disturbance at the boundary may cause a small amount of A to be carried along with the eddy in B, and *vice versa*. The reason for the difference in this phenomenon, with aqueous and alcoholic solutions, has not been investigated.

Fig. 2 (Plate 19) shows photographs of the intertraction observed when a coloured egg albumen solution (s.g. 1·034) was superposed on a sodium chloride solution (s.g. 1·036). The vessel used was a narrow cell about  $1\frac{1}{2}$  mm thick; the albumen shows black. The times given are from the moment of commencing to run the albumen on the surface of the salt. The streamers resemble closely the currents of a liquid descending into, and partly mixing with, a less dense liquid. There is constant swirling motion, the streamers enlarge at the front end and move downward in a rather erratic manner, often changing their course, and sometimes dividing, or even disappearing, when they meet currents of displaced liquid streaming upward (*see* Plates 19–21).

Figs. 3, 4 and 5 (Plates 19–21) show what happens when salt and albumen solutions are mixed in a narrow cell,  $1\frac{1}{2}$  mm. wide, 2 cm. deep, and 7 cm. long, the solutions being introduced separately into the two ends and kept apart till the moment of mixing by a narrow vertical barrier. In fig. 3 the albumen was slightly denser than the salt, and simply flowed underneath it. A few slight irregularities in the surface are due to the vortices of case 3 above. In fig. 4 initially the albumen was of nearly the same density as the salt, and in this case also it flows under the salt without intertraction, but the times show clearly that the flow is slower than when the albumen is initially the denser. Evidently the albumen becomes denser than the salt, through contact with

it, but an appreciable time is necessary for the density difference to become great enough to move the liquids. The slight initial disturbance in the photograph is due mainly to a slight leakage through the barrier before the moment of mixing. In fig. 5, where the albumen is initially less dense than the salt, the albumen begins to run over the top of the salt, but cannot remain there, and streams down rapidly to the bottom. Mixing by the streamers was not quite complete; at the end of 30 minutes the lower 4 mm. of the cell was distinctly deeper in colour than the remainder.

Direct observation of the increase in density of an albumen solution through contact with a salt solution was also made. In one experiment, albumen of  $s.g.$  1.034 was pipetted carefully to the bottom of a beaker containing salt of  $s.g.$  1.032, and removed after two minutes standing. Its density had been increased to 1.0365. In this experiment the albumen was in a layer about 8 mm. deep, and the increase in density represents the average increase through this layer. Evidently much greater increases in density may be possible quite close to the boundary. Calculation from Stokes' law shows that a sphere of  $r$  mm. radius will fall through a liquid of the viscosity of water (taken as 0.0114) at a rate of  $0.383 r^2$  cm. per second, if its density exceeds that of water by 0.002. The front ends of the streamers were generally of diameter 1 to 2 mm., and the rate of descent of some of the fastest streamers was observed, with the albumen and salt, to be about 1.7 mm. per second. Evidently the increase in density necessary to cause the observed rate of descent is easily attainable on the present explanation.

In all, we have used fifteen different pairs of substances, in aqueous or alcoholic solution; these are given with their diffusion coefficients, obtained from Landolt and Bornstein's tables or Jellinek's 'Lehrbuch.' The substances were selected to give a wide range of diffusion coefficients and to be as various as possible in chemical properties. The solutions varied much in concentration; of a pair one was made up arbitrarily, and the other made up in several dilutions so as to give various differences of density. In some cases a pair of solutes was compared at several concentrations. Densities were determined with a small pycnometer without a thermostat and are accurate only to 0.001. In every case, streaming or "intertraction" was observed only when the more slowly diffusing solution was uppermost, and when the difference of density was not greater than about 0.05. From 0.02 downwards it was very marked.

In four cases where the coefficients of diffusion were nearly the same, no streaming could be observed, even when the difference of density was only about 0.005.

It is possible that, by properly taking advantage of this phenomenon, the amount of mechanical stirring required to mix pairs of fluids might be considerably lessened.

*Pairs of Solutions giving Streamers when the more slowly diffusing Solution is Uppermost, the Density Difference being 0.03 or less.*

*Aqueous Solutions.*—

CuSO<sub>4</sub> (0.28) and CoCl<sub>2</sub> (0.5).  
 MgSO<sub>4</sub> (0.3) and CoCl<sub>2</sub> (0.5).  
 CuSO<sub>4</sub> (0.28) and urea (0.92).  
 CuSO<sub>4</sub> (0.28) and KNO<sub>3</sub> (1.2).  
 Albumen (0.06) and NaCl (0.97).  
 KNO<sub>3</sub> (1.2) and HCl (2.3).  
 MgSO<sub>4</sub> (0.3) and HCl (2.3).

*Alcoholic Solutions.*—

Resorcin (0.36) and iodobenzene (0.81).  
 Glycerine (0.35) and iodobenzene (0.81).  
 Resorcin (0.36) and pyridine (0.89).  
 Glycerine (0.35) and pyridine (0.89).

*Pairs of Solutions giving no Streamers, whichever Solution is Uppermost, the Density Difference being 0.004 to 0.009.*

CuSO<sub>4</sub> and MgSO<sub>4</sub>  
 NaCl and urea.  
 Resorcin and glycerine (in alcohol).  
 Pyridine and iodobenzene (in alcohol)

The figures in brackets are the approximate diffusion coefficients, at room temperature.

#### *Negative Surface Tension.*

One of us has recently\* discussed the mechanism of the ordinary capillary phenomena, in order to examine whether there is any evidence that the molecules in the surface of a liquid form themselves into any structure which can be regarded as a contractile skin, producing the surface tension. It was concluded that there is no evidence of such a structure, and that it is improbable, or impossible, without assuming properties of molecules which are unknown

\* 'Nature,' vol. 115, p. 512 (1925).

in other states of matter, whereas it is possible to give a rational explanation of capillary phenomena, assuming no other properties of the molecules than size, attractive force fields, and thermal agitation, if the idea of a contractile skin is not introduced. The mechanisms suggested do not conflict with the idea of a special free energy resident in the liquid and proportional to its surface, and therefore are consistent with the classical theory of capillarity, except where this introduces a mechanical structure in the surface as the seat of the "surface tension"

The mechanism which tends to contract a liquid interface which possesses positive free energy or "surface tension" is that the molecules, being attracted inwards and perpendicularly to the surface by the underlying molecules, leave the surface for the interior, and since the molecules possess size, the surface must diminish. This inward force, at an interface between two liquids, acts to restrain diffusion of the molecules of one liquid into the other. "Negative surface tension" is properly the becoming negative of this inward attractive force on the surface molecules. If it could exist at the free surface of a liquid, it would be a force tending to drive the molecules away from the liquid into the vapour. At the interface between two liquids it would be a force driving the molecules of one liquid into the other, or an attraction of one fluid for the molecules of the other greater than the attraction of these molecules for the other fluid. It is therefore properly manifested as a diffusion of one liquid into the other.

The passage which Schoneboom quotes from Clerk-Maxwell's article\* cannot be considered to be a prediction of the "intertraction" phenomenon, since this is not due to capillary forces, as we have shown. It does, however, show, in a striking way, the difficulties into which one is led, if the surface of a liquid is regarded as being a kind of membrane in tension. After discussing the mathematical expression for the free energy at the interface between two liquids, Maxwell considers what will happen if the expression becomes negative, and concludes that "the displacement of the liquids which tends to enlarge the surface of contact would be aided by the molecular forces, so that the liquids . . . would at length become thoroughly mixed. No instance . . . has been discovered, for those liquids which mix of themselves do so by the process of diffusion, which is a molecular motion, and not by the spontaneous puckering and replication of the bounding surface, as would be the case if  $T$  (the surface tension) were negative." There would be no probability of the "puckering and replication" process, if there were no surface

\* 'Works,' vol. 2, p. 553; 'Ency. Brit.,' Art. on "Capillary Action."

bounding membrane. And if there is no bounding membrane in a liquid diffusion becomes, as we have seen, the normal manner of mixing, as the interfacial tension becomes zero or negative.

*Summary.*

The so-called "intertraction" phenomenon between two solutions only occurs (1) if the solutions are placed one above another and not side by side, (2) if there is a difference in the rates of diffusion between the solutes in the two solutions. Quite different phenomena are observed if (a) the faster, (b) the slower, diffusing solution is uppermost.

Regular streaming as described by Sir A. Wright only occurs if the slower diffusing solution is uppermost, the movements when the faster diffusing constituent is above mainly occur in each layer independently, but eddies may result in a comparatively small amount of mixing of the two layers.

The movements are due to the hydrostatic equilibrium of the layers being upset by the diffusion of the solutes across the interface at different rates. They occur in alcoholic as well as aqueous solutions. Intertraction is not in any way due to capillary forces.

Negative surface tension means that those forces of cohesion perpendicular to the interface, which act when there is positive surface tension to restrain the diffusion of molecules away from the interface, become negative. It is properly manifested in diffusion away from the body of the liquid.

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*Observations on Ipsilateral Contraction and "Inhibitory"  
Rhythm.*

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[PLATES 22-25.]

The following observations on the reflex activities of the vastocruureus muscle in the decerebrate cat have been made by combining, on the same photographic plate, records from a string galvanometer and from a high-frequency isometric myograph. Both methods of investigation have been used separately by numerous workers (1, 2, 3, 4, 5, 9, 10, 11, 13, 14, 16, 21, 22, 23, 25, 28), but it seemed desirable to combine these methods as has been done recently by one of us (18).

*Method.*—The isometric myograph employed (which has been previously described) had a natural frequency of 1,600 per second. Its shadow together with the real image of the string were focussed on the camera by a lens. They were thus both in the same optical system. Silver-chloride electrodes were used as leads, one being inserted into the tendon, the other into the belly of rectus through a "window" made in the skin and sartorius muscle. Sometimes the rectus muscle was dissected away from its fellow muscles in quadriceps and exposed. This eliminated the possibility of bits of skin and fascia touching the proximal lead during the contraction, which was occasionally troublesome, but it had the disadvantage of subjecting the muscle to undue exposure, with resulting deterioration. The stimuli were usually monophasic break-shocks delivered from a Sherrington torsion-wire key. Occasionally double shocks were used, as noted in the legends to the figures. Cats decerebrated under deep anaesthesia by the trephine method were used throughout. To increase the sensitivity, the galvanometer string was made somewhat slack, the tension usually employed being 8 to 11 mm. per m.v. at a magnification of 285.

*Ipsilateral Contractions.*—In a contribution Graham Brown and Sherrington (8, p. 125) have said, "A broad rule observable in the reflex movements elicited from the mammalian limbs by stimuli applied to afferent nerves of the limbs

themselves is that the *movement in the stimulated limb itself is flexion, while that in the crossed fellow limb is extension*. But occasionally this paradigm is departed from . . . and exceptions to this rule have been described by several authors. Duchenne, of Boulogne (10, p. 764), believed from clinical observation that there occurred *associations musculaires modératrices* of antagonistic muscles, but the conception of the reciprocal innervation of antagonistic muscles has ruled out any very active simultaneous contraction of these muscles.

Fröhlich (17) demonstrated simultaneous contraction of extensor and flexor muscles (*antagonistisches Verhalten*) and has compared the reactions to those of the arthropod claw

Sherrington and Sowton (29) and Graham Brown and Sherrington (8) have described a reversal of reflex effect from stimulation of an afferent nerve trunk. Foa (14) obtained electrical records in the chloralized dog of the vastocrureus muscle contracting reflexly from stimulation of the ipsilateral sciatic nerve.

Beritoff and Uchtomsky (4, p. 178) have shown that the rhythm of an inhibitory stimulus is apparent in the inhibited muscle, while Beritoff (2, his fig. 2) has published smoke-drum records of the small ipsilateral contraction of triceps muscle in the frog provoked by stimulation of its inhibitory nerve. Finally, Liddell and Sherrington (24, p. 227) have mentioned the small reflex contraction in vastocrureus muscle elicited by stimulation of the ipsilateral sciatic nerve

Stronger stimuli must be applied to the nerve to produce this contraction of the muscle than are needed to inhibit in it a pre-existing contraction such as that caused by stimulation of the contralateral sciatic nerve. Plate 22, fig. 1A and B, shows the general characters of the ipsilateral contraction. The features that distinguish it from the type of reflex elicited from stimulation of the contralateral sciatic nerve (recruitment reflex) are . (1) short latent period ; (2) rapid rise to a level of tension very much lower than obtained from contralateral stimulation, the ipsilateral tension being seldom greater than 500 gm. ; (3) an early and slight fall of tension ; (4) a clearly marked rhythm even with high rates of stimulation, especially in the early part of the reflex, recordable both by myograph and galvanometer ; (5) comparative absence of after-discharge ; (6) with very strong stimuli it is occasionally twitch-like in character and fails to persist during the period of stimulation (figs. 1B and 12).

The high threshold of stimulation, together with the results obtained by Sherrington and Sowton (28) when they stimulated with repetitive rheonome

currents of low frequency and long duration (slow rise to maximum), suggest that the ipsilateral contraction results from stimulation of a distinct functional group of nerve fibres within the mixed nerve.

*Effect of Tension on the Electrical Response in the Recruitment Type of Reflex* (23).—Cooper and Adrian (11) have described the appearance of secondary waves in the electrical response of quadriceps during a recruitment reflex, especially when the rate of stimulation is low. Each stimulus affects regularly the majority of central nerve units with the production of primary waves. Secondary waves they believed to be due to a small proportion of nerve units responding repetitively and more or less out of phase. Forbes and Cattell (16) have examined the crossed extension reflex electrically, and in the majority of their experiments they were unable to find primary waves corresponding with the frequency of stimulation. It occurred in one of their experiments, but in this they attributed the phenomenon to "escape." In the majority of our preparations (but not in all) we have found primary waves in the crossed extensor reflex provided a low frequency of stimulation was used (15-20 per second), and in agreement with Cooper and Adrian (11) have noted that the secondary waves increase in amplitude and in number as recruitment progresses, as in figs. 2 and 3. The secondary waves not uncommonly mask the primary waves if the stimulus be long continued. The secondary waves also tend to become more apparent as the myographic plateau is being reached, and it is then that the stimulus rhythm in the myograph record becomes less apparent. The effect of increasing the initial tension on the reflex myographic record has been described (Liddell and Sherrington, 22, p. 327) as producing, after a shorter latent period, a steeper rise and a higher development of tension than otherwise occurs.

Combined records made at different tensions show that the initial tension of the muscle is not without its effect on the electrical response. In figs. 2 and 3 are represented records taken at different initial muscle tensions, but otherwise under similar conditions. In 2B and 3B, taken at high tension, there are clearly visible after the third primary wave in the string numerous secondary waves, which are already tending to mask the primary wave, while in 2A and 3A, taken at low tension, primary waves are still clearly visible and secondary waves can be counted only with difficulty, because of their small amplitude on the string. If their amplitude is sufficient to permit counting, there does not appear to be much difference in their number, for a given stage of recruitment, whatever the muscle tension. Again, in fig. 4 the myographic tension is not very great. At the outset, there are only small primary waves visible, and this before there

is any apparent response in the myograph record. Secondary waves begin to appear as recruitment progresses and the primary waves increase somewhat in amplitude. Indeed, from a number of records that have been examined it seems that *the tension of the muscle, whether contraction begins at a high initial tension and therefore responds quickly, or whether it begins at a low tension and therefore responds slowly, is an important factor in the amplitude but not necessarily in the number of the secondary waves*. And when the secondary waves are of moderately large amplitude, as when the tension of the muscle is high (fig 2B), there not uncommonly tends to be an interference or smothering of the primary waves

A factor in the *number* of secondary waves is undoubtedly the length of time of stimulation (Cooper and Adrian, 11). *Ceteris paribus* secondary waves increase in number during a long stimulation, even though there is no great increase in recruitment. Also high frequencies of secondary waves are seldom observed early in recruitment, while late in recruitment (*e.g.*, after 1 second) short periods with frequencies of 200–250 per second are not uncommon. We have often observed that the ascent of the crossed-extension reflex occurs in two steps (figs. 10, 11B and 13). The first step is twitch-like and the string usually under these circumstances shows an initial spike (especially fig 11B). The tension then remains constant for 0.1 to 0.2 second and the recruitment proceeds again, forming the second step

That the rhythm which we have observed in these experiments is not due to "escape" of current is evident for three reasons.—(1) Well-marked string rhythm is invariably reflected by a similar rhythm in the myograph. (2) In a preparation in which we failed to obtain a crossed-extension reflex very strong stimulation did not affect the string. (3) The last stimulus not infrequently shows itself in the string after closure of the short-circuiting key (figs. 8 and 11A).

*Responses of Deafferented Muscle*—In a small number of preparations we have examined the reflex response at different tensions of the muscle before and after its deafferentation by section, intradurally, of appropriate posterior nerve roots. Muscles acutely deafferented have been previously examined by Cooper and Adrian (11), using Magnus' method of novocain injection. It has been our general experience in a preparation examined one to one and a-half hours after posterior root section, that once reflex contraction has begun (and its latent period is usually much reduced) primary waves are less clearly visible, because the string shows (i) "secondary" waves of more rapid onset, and (ii) more considerable amplitude than before, and of (iii) greater frequency, often 300 per second, whereas before deafferentation the responses had

accorded to the usual type. As in the normal muscle, tension appears to be an important factor in the amplitude of the secondary waves.

In fig. 5 (Plate 23) are reproduced responses of the same preparation immediately before (A) and shortly after deafferentation (B). In addition to the differences just noted in the behaviour of the string, it will be seen that the mechanical response shows less tendency toward recruitment, and approaches in general contour the shape of a flexor reflex, but with this difference from a flexor reflex, viz., that in the acutely deafferented preparation the response is characterized by prolonged after-discharge (fig. 5B). In these acutely deafferented muscles which we have examined, the duration of the after-discharge has appeared but little altered as judged myographically, and in the figure reproduced it was actually *more prolonged* after root-section than before.

In the records of 6A and B we have examined the effect of duration of the excitation in the duration of the after-discharge in an acutely deafferented muscle (from same preparation as fig. 5 but on a slower plate), and have found it roughly proportional to the duration of the stimulus. Thus in fig. 6A, when stimulated for 0.51 second, the "plateau" after-discharge lasted 0.2 second, while in 6B with a stimulus duration of 1.81 second the "plateau" after-discharge was, approximately, 0.52 second in duration. The curious step-like relaxation shown in fig. 6A has occurred several times in deafferented preparations, and the reason for its occurrence is not clear.

The activity of the string is little diminished in the earlier part of after-discharge, and it subsides slowly as the muscular contraction comes to an end.

*Inhibition of Crossed Extension Reflexes*—Liddell and Sherrington (24, 25) have described the characters of inhibitory processes as examined with the high-frequency myograph, while Beritoff and others (2, 4, 13, 14, I) have described electrical changes during inhibition. In our present experiments we have found the high-frequency myograph lever to be a valuable guide to the effect of the inhibitory stimulus, when investigating electrical changes under varying conditions. At the onset of inhibition the string reacts rather more rapidly than the myograph, but if the myographic tension is at that moment high (e.g., 3 kg) the difference in time between the two records is often no more than 0.05 to 0.06 second (fig. 7, Plate 24), which would be only slightly longer than the after-action (19) of a motor-nerve tetanus at that tension (Liddell and Sherrington, 22).

In our experience, when the repetitive inhibitory stimulus is strong—that is, when the myograph lever falls rapidly towards the base line—the string shows

the rhythm of the inhibitory stimulus from the onset of inhibition (fig. 7A). The amplitude of the string also is greater at the onset of inhibition, when the number of active nerve units and the mass of active muscle are greater. When, however, the inhibitory stimulus is less strong (fig. 7B) and the myograph lever falls slowly, showing the step-like process of negative recruitment, the string responds only to alternate inhibitory stimuli—as, indeed, does also the myograph. This alternate response can sometimes be elicited when the inhibitory stimulus, although breaking through only in alternate waves, is, nevertheless, strong enough to provoke rebound. In fig. 8 the inhibitory rhythm breaks through at the onset at only one-half its full rate, later one-third, and then, finally, at its full rate as inhibition becomes complete. The interpretation of fig. 9 would seem to be that in some cases with moderately strong inhibition both rhythms may be seen in the string, as Beritoff (4 and 5) has shown, and even in the myograph (Liddell and Sherrington, 22 and 24).

In another record there appeared at first the full inhibitory rate, which later becomes irregular or formed into double groups, finally giving the full rate, and as well the excitatory rhythm for two or three waves at the beginning of inhibition. The waves show themselves on the string as brief periods of damping.

The comparatively stronger stimuli that are necessary to produce complete inhibition are strong enough to elicit the ipsilateral contraction, which in the trough of inhibition shows myographically by the small rhythmical residual contraction and galvanometrically by vibrations at the inhibitory rate (fig. 7A). It is not until the inhibition has come to an end (Liddell and Sherrington, 24) that the myograph lever shows complete relaxation, denoting the fully inhibited state, and only during the same "after-period" of inhibition does the string show complete quiescence. Before the onset of stimulation (figs. 7A and 8) the string may have shown the small vibrations of Buytendijk (9); and Einthoven (13), but in the "after-period" of inhibition, when the ipsilateral contraction has ceased, the string is in complete rest (figs. 7A, 11A and B) [*conf.* (1), (3), (12), (13)]. The duration of the after-period is variable and is, as a rule, directly affected by the strength of the inhibitory stimulus, being longer with stronger stimuli. In fig. 7A its duration is less than 0.05 second; in 11A it is 0.12 second, while in 11B, with a very strong stimulus, it has lasted 0.36 second. From this it is evident that the effect of the inhibitory stimulus lasts over a considerable period (*cf.* Beritoff, 5), but the after period seems to be cut short often by the onset of "rebound," which itself is a capricious phenomenon (fig. 7A, fig. 12) (Sherrington, 27; G. Brown, 6 and 7).

In a small number of cases, we have employed single break shocks as agents for inhibition. The myographic reactions under these conditions have already been described (Liddell and Sherrington, 25, and Sherrington, 26). In fig. 10 is recorded the effect of a fairly strong single break shock given as an inhibitory stimulus during the progress of recruitment. Its effect on the myograph is to produce a fairly rapid decline of step-like negative recruitment, the steps of which correspond to the rate of the excitatory stimulus. On the string, the effect is an enhancement of at least four primary excitatory waves, with a depression of secondary waves, whereas before the inhibitory break shock, primary waves had not been clearly marked and secondary waves had been numerous. In other records with very strong single stimuli appears depression of both primary and secondary waves.

In fig. 11A and B (Plate 25), intercurrent tetanic inhibition has been produced during constant contralateral stimulation. The myographic recovery after inhibition is not steep and shows the stimulating rhythm clearly. It can therefore be presumed to be free of "rebound". In correspondence with these "bare ribs" in the myograph record it is seen in the galvanometric record that there is a comparative absence of secondary waves, and the primary waves have not the same amplitude as in the original recruitment.

In fig. 12, a record of a muscle not entirely deafferented, there is evidence of some autogenetic inhibition during recruitment and of rebound from this inhibition at the cessation of the stimulus. It is apparent that during this period of autogenetic inhibition there is a decrease in the amplitude of the secondary waves. It appears from this possible that the autogenetic inhibition is responsible for the two-stepped ascent which is often encountered in the crossed extension reflex.

*Rebound* —When the intercurrent inhibitory stimulus is sufficiently strong, in a favourable preparation, to produce rebound, as judged by the great and rapid increase in the tension of the muscle, there occurs a great increase in the amplitude and possibly the number of secondary waves, with a masking of primary waves.

Rebound can be elicited in an otherwise unstimulated but only tonic muscle by applying inhibitory stimuli to the ipsilateral sciatic nerve. The rebound that ensues after the withdrawal of the inhibition is recorded in fig. 12. At the onset of inhibition a twitch-like ipsilateral contraction appears. That is followed, in this preparation, by inhibition of the pre-existing tonic tension, while the string shows only the inhibitory rhythm. Very soon (0.04 sec.) after the withdrawal of this stimulus, "rebound" appears, giving in less than 0.6 sec.

a tension of nearly 4 kg. The string shows waves of a frequency of, often, 250 per sec. and of a relatively large amplitude. There is no regular movement of the string and no trace of the previous inhibitory rate.

*Experiments with strychnine*—After the intravenous injection of a dose of strychnine just below the "reversal" dose (0.08 mgr. per kilo.) stimulation of the contralateral sciatic nerve produces a recruitment showing very clearly stimulus rhythm in which there is no longer a smoothing over from one step to another to give the usual type of myogram. Instead, the steps are very marked (fig. 14). In correspondence with this, the string shows large primary waves and relatively few, but large, secondary waves. Occasionally secondary waves after strychnine appear altogether absent\*. Inhibition, after this dose of strychnine, is more often than not complete and rapid provided the inhibitory stimulus is not too strong, and during this time both the myograph and the string show inhibitory rhythm. When the inhibitory stimulus is withdrawn, the recruitment begins rapidly again. In it the steps of the stimulus rhythm are even more marked than before (a phenomenon similar but greater in degree than in the normal preparation). In correspondence, there are fewer secondary waves recorded by the string. The inhibition may still leave a depressant effect, even 0.10 sec. after its withdrawal, as has been noticed also in records from the non-strychninised preparation. Thus, occasionally we have inadvertently stimulated the inhibitory nerve by a single break shock *before* the excitatory nerve was stimulated, and have noticed that response was considerably smaller than when it had not been preceded (2 to 3 sec.) by an inhibitory shock. This is in keeping with Sherrington's (26) statement that inhibition is a condition of the cord which can exist independently of excitation.

#### *Discussion.*

The increased size of the secondary waves when the response commences at a high initial tension is in keeping with previous observations of Buytendijk (9), who found that the waves in decerebrate rigidity became augmented when the tonic muscle was put under stretch, and more recently with those of Forbes and Cattell (16), who amplified the electrical responses from a tonic gastrocnemius preparation and found a considerable increase in the size of the electrical responses with increased tension. As one of us (18) has previously found in the frog that tension increases the size of the electrical

\* Our friend, Dr. F. Bremer, of Brussels, so he kindly informs us, has obtained in collaboration with Dr. P. Rylant results similar to our own, which will be published shortly.



responses of the individual muscle fibre stimulated through its motor nerve, we have been inclined to regard the increased size of the secondary waves with tension as purely a peripheral effect. In an investigation now in progress we are finding that the electrical responses of mammalian muscle stimulated by the motor nerve behave as does frog muscle (18 and 19) to different mechanical conditions

We have interpreted the behaviour of the string during inhibition as follows. When a short repetitive inhibitory stimulus of high intensity is given during a crossed extension response, the inhibitory rhythm, as we have seen, may be observed from the outset of the inhibition, and this we regard as due to the small uninhabitable increment of ipsilateral contraction (*cf* Adrian, 1, p. 407), for the rhythm may also be seen in the myograph. Inhibition under these circumstances appears to be complete only after the inhibitory stimuli have ceased, when one observes a period of complete quiescence in the string as well as the so-called post-inhibitory notch in the myograph (figs 7A, 11A and 11B). When inhibition is elicited during crossed excitation by a single break-shock a number of primary excitatory waves become enhanced, due to the suppression of secondary waves. We have interpreted this, according to the view of inhibition which has recently been put forward by Sherrington (26) and by Liddell and Sherrington (25), as indicating that the single break shock suppresses the repetitive after-discharge in a large number of central motor units, with the result that primary waves, as in the beginning of a response, are able to make themselves evident. This renders more simple the understanding of weak repetitive inhibition in which both excitatory and inhibitory rhythm may be seen in the string. Such an inhibitory stimulus appears to recruit itself by diminishing first the after-discharge in the increasing number of central units. This causes in early stages of weak inhibition the unmasking of primary excitatory waves which are seen especially after a single break shock inhibition. As the negative recruitment proceeds, the primary waves become diminished, and, finally, a stage may be reached in which only the ipsilateral contraction remains, when one will see only the full inhibitory rhythm. The explanation of the phenomenon occasionally observed (fig. 7B) that only every second or third inhibitory stimulus appears in the string is not at present clear, but may be accepted as further evidence of the summation of stimuli in reflex processes.

We have adopted the view discussed by Cooper and Adrian (11) that secondary waves are due to repetitive asynchronous after-discharge. In the light of this, our deafferentation experiments would appear to show that when

the spinal centres of quadriceps are "released" from their proprioceptive supply (1) recruitment occurs more quickly, (2) repetitive after-discharge is more pronounced, as judged by the rapid onset of secondary waves and corresponding absence of rhythm in the myograph, (3) terminal after-discharge is as long and may be longer in a deafferented than in a normal preparation. This suggests that a factor in proprioceptive co-ordination is the prevention (by autogenetic inhibition) of too rapid onset of responses of reflex or central origin.

Finally, we are in agreement with Forbes and Cattell (16) that the frequency of the string vibrations is no real index of rates of discharge of the individual motoneurons of the spinal centres, and our observations are in accord with theirs from which they have inferred that in the crossed extension reflexes the responses of the individual elements are in great part out of phase, with the result that the action currents are small in comparison with the large number of units recruited. In after-discharge and in rebound the action currents are similarly asynchronous.

#### *Summary.*

Simultaneous mechanical and electrical records have been obtained (with string galvanometer and torsion-wire myograph of high frequency) of responses of quadriceps extensor muscle (cat) to various forms of reflex stimulation, before and shortly after section of posterior root supply of the muscle.

When the normal muscle is reflexly stimulated at 50 per sec. through sciatic nerve of same side, a small rapidly developed contraction ("ipsilateral") results, in which the rhythm of stimulus may be seen in both string and myograph.

Crossed stimulation at 15 to 20 sec. before cutting the posterior roots produces a response, in which during the mechanical ascent (i.e., during recruitment) the rhythm of the exciting stimulus may be seen in the string ("primary" waves) and the mechanical record, but later in response both rhythms tend to be obliterated through the appearance of increasingly large numbers of "secondary" waves.

After cutting the posterior roots the secondary waves are more numerous from the start, and both string and myograph show a corresponding absence of primary rhythm. In acutely deafferented muscle mechanical response develops more rapidly, and duration of terminal after-discharge is sometimes longer than in normal muscle.

Increase in mechanical tension increases amplitude of both primary and secondary waves.

A single moderately strong break-shock, inhibition during a crossed extension response causes, through suppression of secondary waves (repetitive asynchronous after-discharge) an enhancement of primary excitatory rhythm in both string and myograph.

Repetitive inhibition, if weak, produces the same effect; if strong, it gives rise to a rhythm of its own rate in both records. This "inhibitory" rhythm during "complete" inhibition we interpret as due to the small uninhabitable increment of ipsilateral contraction, for it is followed by a period of complete quiescence and by the corresponding post-inhibitory notch of the myograph. Duration of post-inhibitory quiescent period increases with strength of inhibition.

#### DESCRIPTION OF FIGURES

##### PLATE 22

FIG. 1A.—Ipsilateral reflex contraction of quadriceps (cat) caused by stimulation of the sciatic nerve. Time, above 0.02 second. Short-circuiting key below time signal shows duration of stimulus. The initial upward movement of the string was caused by the skin touching the proximal electrode during the response. The rhythm of stimulus is clear in both the myograph and the string. Zero tension is shown by black line just below the myograph. At bottom of plate is the vibrator showing the rhythm of stimulus (50 per second).

FIG. 1B.—Ipsilateral contraction of another preparation (same as that from which fig. 1A was taken) stimulated at 14 per second, and with a higher intensity of stimulation. This shows the initial twitch followed by gradual recovery. It may be noted that the size of the electrical variations increases with the mechanical, showing that movement of the string is not caused by escape of current.

FIG. 2A and B.—Crossed extension reflexes taken at different initial tensions of the muscle to show alteration in magnitude of secondary waves. Stimuli, 14 per second at same strength in both responses. Time, 0.02 second. Upper signal, short-circuiting key. (A) Initial tension, 100 gm.; (B) Initial tension, 420 gm.

FIG. 3A and B.—Same as fig. 2 from another preparation. In this the primary waves are smaller, which is reflected in the myograph by rhythm of stimulation being less marked.

##### PLATE 23.

FIG. 4A and B.—Weak crossed extension reflex stimulated at 18.5 per second with concurrent inhibition at 50 per second. Moment of excitation not signalled, period of inhibition shown between the two middle signals. Rhythm of excitation, upper signal; that of inhibition, lower signal. Time, 0.02 second. The small number of secondary waves during recruitment may be noted, as well as the inhibitory rhythm which is followed by a prolonged quiet period. In both cases the last "inhibitory" response occurs after the "off" key had closed, showing that the inhibitory rhythm is not due to escape of current.

FIG 5A.—Crossed extension reflex *before* severing posterior roots. Rate of stimulation, 14 per second, duration of excitation indicated by short-circuiting signal below line of zero tension. Note distinctness of primary waves and the rhythm in the early part of the ascent of the myograph. The slight extraneous movement of the string was due to electrode movement

FIG. 5B.—The same reflex of the same preparation two hours *after* cutting, intradurally, the posterior root supply of the muscle. The same strength, duration and rate of stimulation used in both, and the initial tension of the muscle was approximately equal in the two responses. There is a slight initial extraneous movement of string, and a complete or nearly complete absence of primary waves, with a corresponding lack of rhythm in the myograph. Contrast the shape of the mechanical response with that in 5A; compare also the respective durations of after-discharge

FIG 6A.—Same as 5B (*i.e.*, deafferented), but taken on a slower plate. In this there is some evidence of primary waves, but the secondary waves are very numerous from the start. Duration of excitation 0.51 second, and of "plateau" after discharge approximately 0.20 second

FIG 6B.—Same as 6A, but in which excitation lasted 1.84 second, and the "plateau" after-discharge was in this case 0.52 second in duration

#### PLATE 24

FIG 7A.—Crossed extensor contraction with concurrent strong inhibition (coil, 10 cm.) From above downwards are: time signal, 0.02 second, vibrator showing excitatory rhythm (15.5 double 31 per second); inhibitory short-circuiting key, galvanometer string, myograph, line of zero tension, excitatory short-circuiting key, vibrator showing rhythm of excitation (50 per second monophasic). Note inhibitory rhythm of string followed by a short quiet period corresponding with the post-inhibitory notch in the myograph. String tension, 11 mm per m.v. at magnification of 285

FIG 7B.—Same reflex under the same conditions, taken two minutes after the preceding, with a weaker inhibition (coil, 16 cm.) Excitation the same strength (coil at 9 cm. with 25,000 ohms graphite resistance in secondary). Half the inhibitory rhythm has "come through," *i.e.* 25 per second, both in the string and in the myograph, and there is no post-inhibitory notch.

FIG 8.—An inhibition similar to that shown in fig 7B, in which, during the early part of the inhibition, every other inhibitory stimulus has appeared in the string. This is followed by a brief period in which every third inhibitory stimulus appears, and finally at the end of the inhibition the full inhibitory rate of 50 per second can just be seen. The inhibitory vibrator is not vibrating in this figure.

FIG. 9.—A weak inhibition (coil 15) similar to the last, but in which the predominant rhythm during the inhibition is that of excitation, though there is some evidence of the inhibitory rhythm as well.

FIG 10.—An inhibition evoked by a moderately strong single break-shock at a point shown by the signal. The result of the inhibition is an enhancement of four primary excitatory waves, with a diminution of secondary waves.

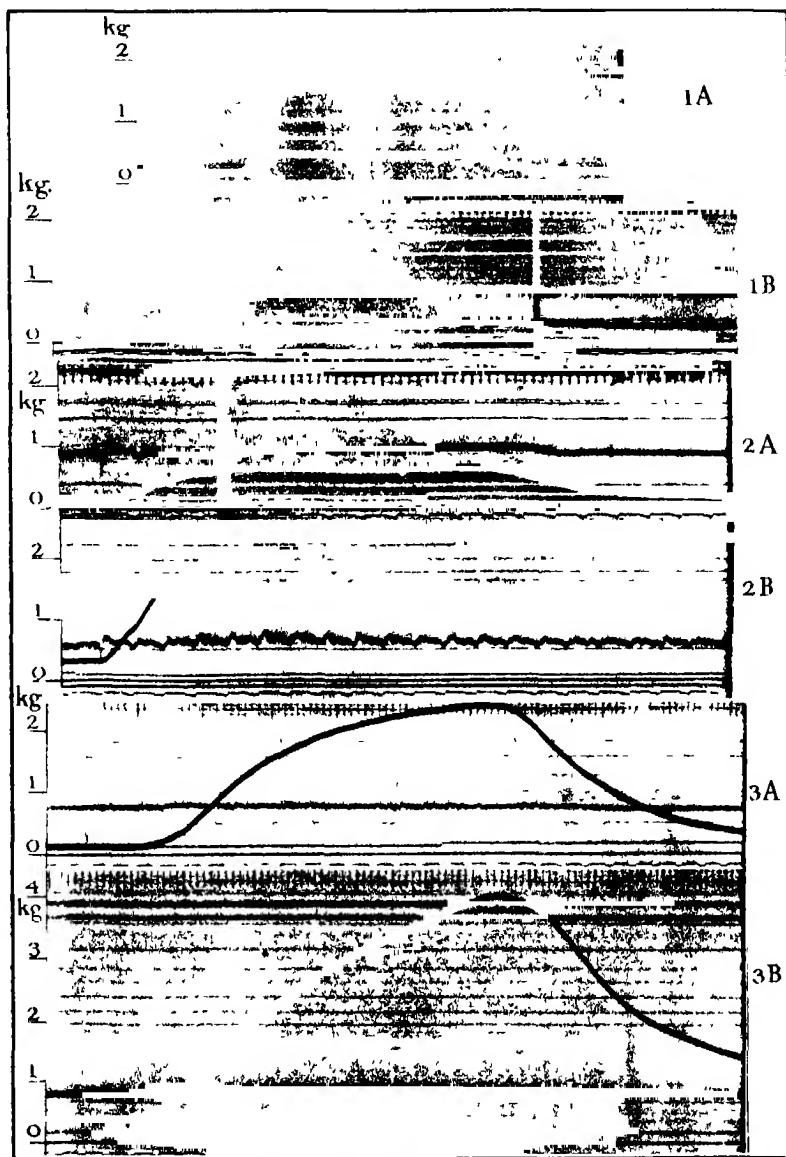
#### PLATE 25.

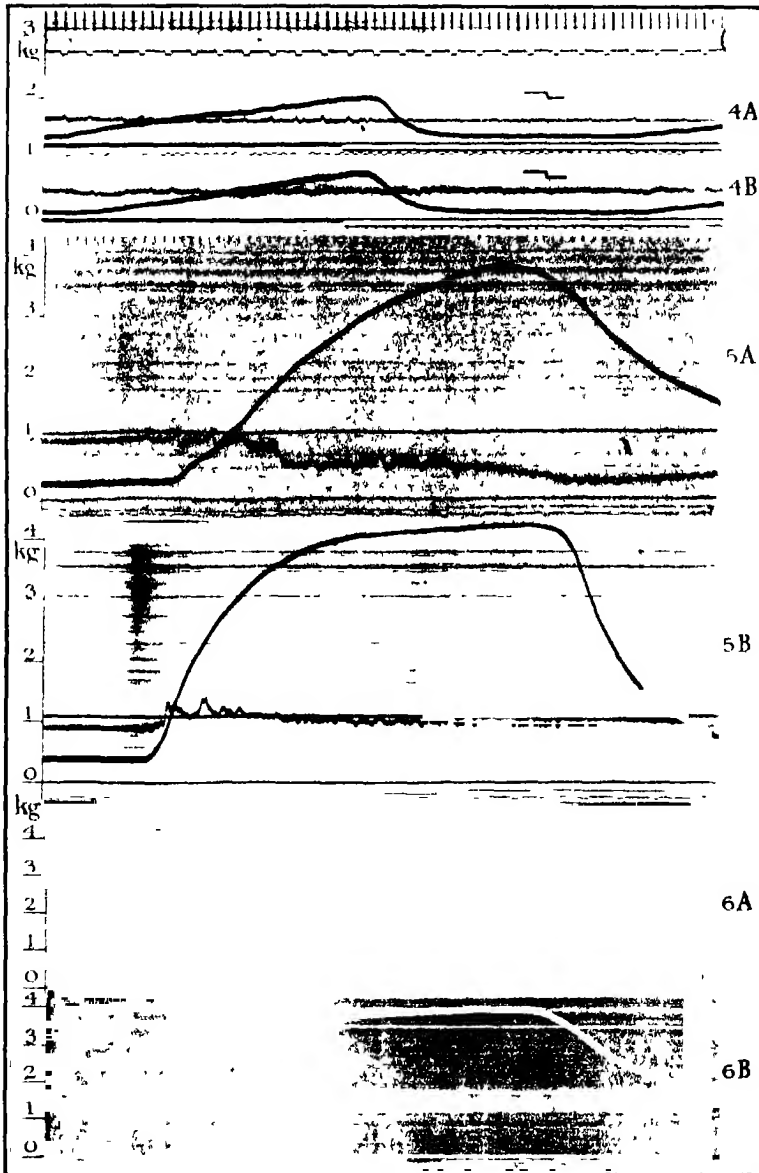
FIG 11A.—Inhibition of crossed extensor reflex showing post-inhibitory notch with "quiet period" of string and redevelopment of response without evidence of rebound. Note primary waves with increasing numbers of secondaries after the inhibition.

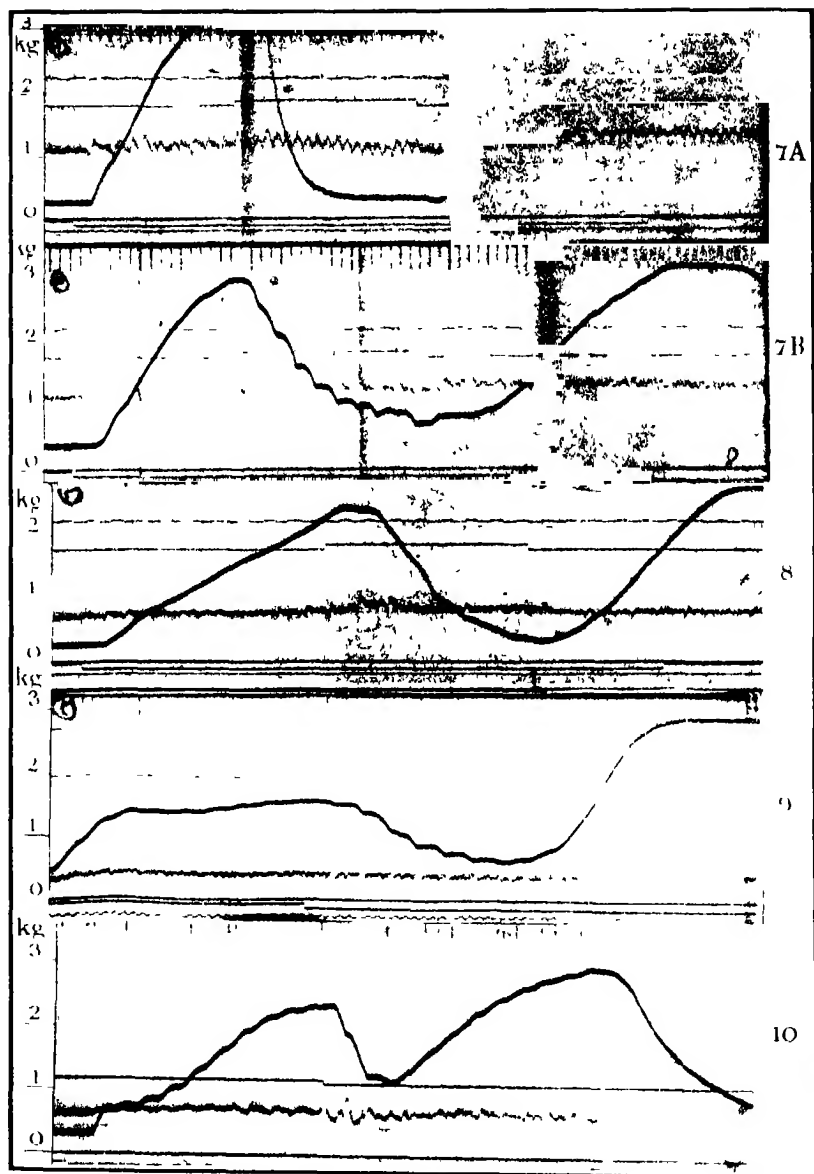
- FIG. 11a.—Same preparation with somewhat more effective excitation (coil as in 11a at 12 cm.), owing presumably to the nerve being in better condition. Inhibition stronger. Note greatly increased duration of the post-inhibitory notch and "quiet period." There is evidence of a slight degree of autogenetic inhibition in this ascent.
- FIG. 12.—An ipsilateral contraction followed by rebound. This was taken immediately after the ipsilateral contraction recorded in fig. 13. Rate of stimulation, 14 per second (monophasic).
- FIG. 13.—Crossed extension reflex of a partially deafferented muscle showing two-stepped ascent followed by a rebound after cessation of stimuli. In post-mortem examination it was found that a fasciculus of the fifth lumbar posterior root remained uncut. Time not indicated, but it can be calculated from rhythm of stimuli (14 per second).
- FIG. 14.—A crossed reflex with concurrent inhibition after a small dose of strychnine. Note the size of the primary waves and the corresponding ribs in the ascents.

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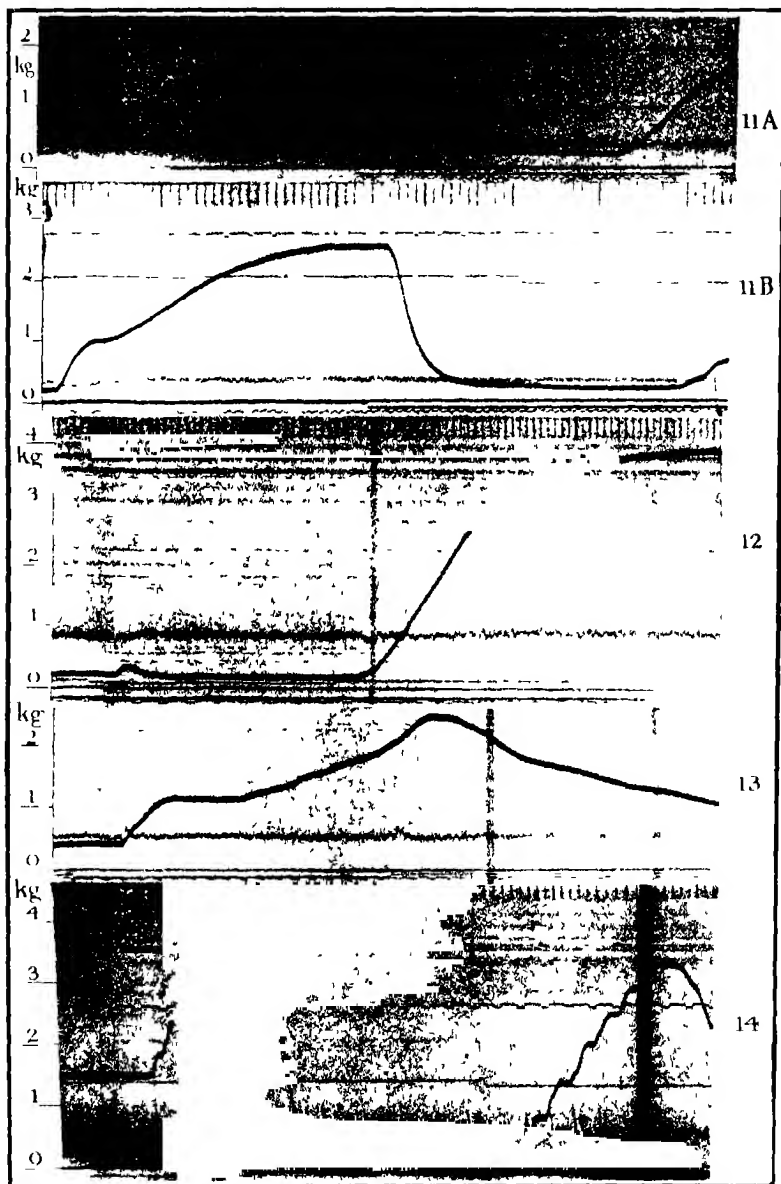
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*The Nucleolus of Tmesipteris Tannensis, Bernh.\**

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[ PLATE 26 ]

In some slides from young synangia of *Tmesipteris*, prepared from curiosity regarding this unique plant, it was noticed that during mitosis there were present, in addition to the chromosomes, several rounded bodies similar to the nucleoli of resting cells. Moreover, nucleoli were present in nuclei where the spireme was fully developed prior to mitosis. Preliminary examination thus suggested *Tmesipteris* as a most favourable plant for a study of the nucleolus as regards its relationship to the chromosomes and its behaviour during mitosis.

*Historical.*

The study of plant-nucleoli has been largely incidental to the investigation of chromosome behaviour, more especially during the meiotic divisions. The general conclusions from the study of plant-nucleoli may be summarised thus :— The number of nucleoli per cell may vary from one up to eight or ten, some of which may arise by the division of pre-existing ones. (1) In some cases they have been regarded as stores of material which contribute to the building of the chromosomes in prophase (2, 3), while in other cases this explanation has been rejected. Another view, supported by Strasburger and Chamberlain (4, 5) amongst others, is that spindle-formation is a function of the nucleoli. Others

\* Sahni ('Phil. Trans.,' B, vol. 213) distinguishes in the species *Tm. tannensis* the two varieties *elongata* and *lanceolata*, in addition to the type. It seems doubtful if these are distinct forms. On *Cyathea dealbata* under suitable conditions of shade and moisture, *Tmesipteris* appears always to take the form of Dr. Sahni's variety *elongata*.—(J. S. Y., 28.4.25.)

again hold that extruded portions of the nucleolus are converted into organs such as the blepharoplast of *Hepatica*. Extrusion of chromidia into the cytoplasm by nucleoli has been described by various cytologists (6, 7), and Digby (1) finds that the nucleolus of *Gallonia* is closely related to one pair of chromosomes, and at metaphase is "pushed" away from the equatorial plate on the end of a chromosome. Lack of genetic continuity between nucleoli has been advocated by many authors, especially by Gates (8, 9, 10) and by Mottier (11).

The nucleolus of animals has recently yielded many interesting results, which must be taken into account in any work on plant-nucleoli. Gatenby (12) and Hogben (13) have shown that fragments of the nucleolus may give rise to secondary nuclei, exactly simulating in appearance real nuclei. Carleton (14) has shown that during mitosis in the frog, the "nucleolus" divides and that one part of it is found in each daughter nucleus. Such behaviour on the part of the nucleolus has led to the view expressed by Carleton (*loc. cit.*) and more specifically by Gatenby (15) that the nucleolus may be a self-perpetuating body and that it is independent of the chromosomes.

On the other hand, Stevens (16, 17) has shown that in many insects the sex-chromosomes during the meiotic phase are regularly and intimately connected with the plasmosome. In marsupials Agar (18) and Greenwood (19) have shown that the same relationship obtains, and Agar has mentioned in addition, that the plasmosome seems to be extruded from the combined sex-chromosomes. A short discussion of the above views will be taken up, following an account of the nucleoli in *Tmesipterus*.

#### *Materials and Methods.*

Almost the whole of the material used was obtained from plants growing on the tree-fern *Cyathea dealbata*, in the neighbourhood of Wellington. Synangia and growing-points (aerial stem and rhizome) were used. Fixation was carried out between noon and 3 p.m. during all seasons of the year. Fixatives used include the following:—Bouin's picro-formol-acetic; chromacetic, corrosive sublimate (sat. aq. soln.); the same with 5 per cent. of acetic acid ("corrosive-acetic"); Fleming-without-acetic (Gatenby); Altmann, Regaud's formol-bichromate; formalin (1 in 9).

Dehydration was effected by slowly-graded alcohols, by a drip method, or by the glycerine method used by Digby (20). The material was cleared in xylol or cedar oil, embedded in 52° C. paraffin, and cut at thicknesses from 4 microns to 22 microns. The stains most used were Heidenhain's iron-haematoxylin, thionin blue-eosin, Auerbach's stain, methyl blue-acid fuchsin, methyl green

with 1 per cent. acetic acid (combined fixing and staining). Iron-hæmatoxylin was the most generally useful stain, though the simultaneous stains were invaluable in distinguishing nucleoli from chromosomes.

Both Auerbach's stain and the methyl blue-acid fuchsin (equal volumes of 1 per cent. solns.) mixture were found to give good results only after fixation in corrosive sublimate mixtures. The latter staining-mixture seems preferable to the Auerbach's, for in it the chromosomes and cytoplasm take the blue stain, the nucleoli alone being red.

For the gametophyte material of *Tmesipteris* which was used in this work I am indebted to Dr. J. E. Holloway, who had collected it for his work on the gametophyte and the young sporophyte (21). The fixative used was weak chromacetic.

#### *The Nucleolus of the Sporophyte.*

A most striking feature in the cytology of *Tmesipteris* is the size of the nuclei, which have often a diameter of 25 microns. While the size is an advantage in many ways, yet it is disadvantageous in that it is not convenient to use sections thick enough to contain even a proportion of uncut nuclei.

The appearance of the nuclear reticulum varies considerably according to the fixative used and to the tissue in which the nucleus is situated. In meristematic regions the reticulum is fairly light, with pronounced peri-nucleolar vacuoles. In cells of the synangium wall, the nuclei are smaller, their reticulum dense and deeply-staining, while peri-nucleolar vacuoles are almost lacking. In resting nuclei the nucleoli are generally rounded in shape and smooth in outline (Plate 26, figs. 1, 5b). Slight irregularities of outline are seen where the nucleoli are in connection with the reticulum. Very rarely a dumb-bell shaped nucleolus is seen in resting cells, indicating, in some cases, a division of the nucleolus (Plate 26, fig. 5a). The fixing and staining reactions of the nucleoli leave no doubt that they are plasmosomes. Acid-methyl green fixation stains the chromosomes but leaves the nucleoli almost colourless. Iron-hæmatoxylin washes out from the nucleoli more slowly than from any other part of the cell. Both in Auerbach's stain and in the methyl blue-acid fuchsin mixture the nucleoli stain red.

After fixation in corrosive sublimate, formalin, and all the acid fixatives save acid-methyl green, the nucleoli appear homogeneous except for occasional small vacuoles. Following the acid-methyl green mixture, the nucleoli appear to contain a varying number of small, refractive globules. In some nucleoli these fuse to produce one or more large refractive bodies. Nucleoli of material fixed in Regaud and in Fleming-without-acetic, stained in iron-hæmatoxylin,

have the appearance shown in Plate 26, figs. 7a, 7b, 7c. This structure is characteristic of meristematic tissues, and is brought out best by Regaud's method. Associated with it is a plain increase in the number of mitochondria shown in the cytoplasm of developing synangia by the same method. It seems likely that these mitochondria originate from the nucleoli and pass out through the nuclear membrane.

The number of nucleoli in each section (8-10 microns) of a nucleus may be anything up to five or six. To determine accurately the number in each complete nucleus, the following method was used:—

An area of nuclei in nine successive serial sections of a young synangium (Boun-Heidenhain; cut at 8 microns) was outlined under camera lucida at a magnification of 480 diameters. Nucleoli were marked in each nucleus at the same time. By making the drawings very bold on foolscap paper, those of adjacent sections could be superimposed and the nuclei in them followed with certainty in all their sections. The individual nuclei and their nucleoli were then treated in the same way except that they were drawn at a magnification of 3000 times. By this method not only were all the parts of each nucleus identified, but the occasional sectioning of nucleoli could also be seen, practically eliminating this source of error. Moreover, there could be no unconscious selection of nuclei which agreed with the observer's preconceived ideas, for every nucleus in a certain area was studied.

Of 62 nuclei examined in this way, 45 had each five nucleoli, 10 had six each, 6 had four each, and one alone had three nucleoli—a total of 314 nucleoli to 62 nuclei. Although the nucleoli are not quite regular in shape (Plate 26, fig. 1), yet it seemed that the accuracy was sufficient to justify an attempt at correlation between the number and the size of nucleoli, and the size of the nucleus. In Table I are given the mean diameters of 28 nuclei and their nucleoli, the dimensions being obtained from outlines at a magnification of 3000. Under the heading "Area in Section" are given the square of the mean diameter of each nucleus (in centimetres from outlines  $\times$  3000) and the sum of the squares of the mean diameters of its nucleoli (in millimetres). The nuclei are arranged in descending order of size, and the nucleoli of each one across the page in order of size.

It will be seen from Table II that the average sectional area of the nucleoli in the 14 larger nuclei is about 20 per cent. greater than that of the nucleoli in the 14 smaller nuclei. Such a difference is certainly greater than possible errors in measurement, errors which in such a number of measurements (147 nucleoli) would tend to neutralise one another. Table I shows with reasonable

Table I.

No.	Dia- meter of Nucleus (microns)	Diameters of Nucleoli.						Area in Section	
		1	2	3	4	5.	6	Nucleus.	Nucleoli
1	26.6	5.0	4.4	3.3	—	—	—	cm <sup>2</sup> 64	mm <sup>2</sup> 474
2	26.3	3.3	3.3	2.6	2.6	2.3	—	62	377
3	25.6	3.3	3.0	2.6	2.6	2.6	2.3	52	422
4	25.6	4.6	3.6	3.3	2.6	2.3	—	59	530
5	25.0	4.6	3.6	3.0	2.6	2.6	2.3	56	515
6	25.0	3.3	3.3	3.0	3.0	2.6	2.3	56	475
7	24.6	4.5	3.3	3.3	3.0	2.6	—	55	527
8	24.6	3.3	3.3	3.0	2.6	2.3	6.0	55	428
9	24.3	4.6	4.3	3.0	2.6	—	—	53	510
10	24.0	4.6	3.6	3.3	3.3	3.0	—	52	598
11	23.6	4.2	3.3	3.3	2.6	1.6	—	50	445
12	23.1	3.6	3.3	2.6	2.6	2.3	—	49	398
13	23.1	3.3	3.0	3.0	2.8	2.6	2.3	49	447
14	22.6	3.6	3.3	2.6	2.6	2.3	—	46	398
15	22.3	4.3	3.0	2.6	2.3	2.0	—	45	399
16	21.6	3.6	2.6	2.3	2.3	2.0	—	42	334
17	21.6	4.0	3.0	3.0	2.6	2.3	—	42	419
18	21.6	3.6	3.3	3.3	2.6	2.6	2.3	40	498
19	21.0	3.3	3.3	3.0	2.6	2.3	2.0	40	424
20	21.0	3.3	3.0	2.6	2.6	2.0	2.0	40	381
21	21.0	4.2	3.3	2.6	2.3	2.3	—	40	435
22	21.0	3.6	3.3	2.6	2.3	2.3	—	40	383
23	21.0	3.3	3.0	2.6	2.6	2.3	—	40	358
24	21.0	3.3	3.0	2.6	2.6	2.3	2.0	40	394
25	20.6	4.2	3.3	2.6	2.3	2.3	—	38	435
26	18.3	3.6	3.3	3.3	2.3	2.0	—	30	406
27	18.3	3.6	3.0	2.3	2.3	1.6	1.6	30	350
28	18.0	4.0	3.0	2.3	2.3	2.0	—	29	365

Table II.

	Nos 1-14	Nos 15-28.
Sum of the areas of nuclei	712 cm <sup>2</sup>	536 cm <sup>2</sup>
" " " nucleoli	6544 mm <sup>2</sup>	5581 mm <sup>2</sup>
Number of nucleoli	72	75

certainty that within these limits of nuclear size, the number of nucleoli is independent of the size of nuclei. The numbers 72 and 75 in Table II are as close as could be expected. Moreover, each group of 14 nuclei has five which contain each six nucleoli. The number of cells containing three and four nucleoli (one of each) is too low to allow of any conclusions being drawn.

Table I shows a close relationship between the number of nucleoli in a cell

and their size. The average diameter of the chief nucleolus in each of the 16 cells containing five, is four microns, that of the corresponding nucleoli in cells containing six, 3.5 microns, higher numbers being thus accompanied by a lower size. The occurrence of the largest nucleolus in the table (5 microns) in a nucleus containing only three, supports this conclusion.

Table II shows fairly conclusively that the number of nucleoli is independent of the size of the nucleus, and this point needs no further attention at present. On the other hand, it seemed desirable to ascertain whether the relationship between the sizes of nuclei and of their nucleoli were constant for all kinds of tissue, *i.e.*, whether small nucleoli always accompany small nuclei and *vice versa*. Tracings were made at 3000 diameters as for Table I. In Table II are the results of the measurement of ten separate lots of nuclei. Number 1, 2, 3 are from Bouin material, 4 and 5 are from corrosive-acetic, the remainder from chromacetic material. Iron-haematoxylin was used throughout. Brackets indicate that the nuclei are from the same slide.

Table III —Nucleoli in Resting Cells

Lot No	Position in Plant	No of Nuclei examined.	Average diameter of Nuclei.	Average diameter of Nucleoli	Ratio of Diameters
			microns	microns	
1	Sporogenous tissue	31	23.0	3.0	7.6
2	" " (prophase)	14	26.7	3.4	7.8
3	Synangium wall	20	17.3	2.6	6.6
4	Rhizome near tip	20	18.8	2.13	8.8
5	" growing point	20	24.7	2.8	8.7
6	Synangium wall	20	16.9	2.3	7.3
7	Sporogenous tissue	12	25.1	3.0	8.4
8	Stem, near tip	20	21.2	2.4	8.7
9	Prothallus near apex	41	13.7	1.73	7.8
10	Egg nucleus	7	23.2	3.7	6.2

It will be seen that, with qualifications, large nucleoli are associated with large nuclei and *vice versa*. The marked increase in size of both at prophase is especially noticeable. Also it is worthy of note that nucleoli of the synangium wall are, by comparison with their nuclei, larger than other nucleoli from the same slide. The results for Lot 10 are not reliable, for the egg-cells were old and brown and somewhat shrunken.

It is difficult to place any satisfactory interpretation on the results of Table III. The larger nuclei (Lots 1, 2, 5, 7) are actively growing and dividing, and the larger size of the nucleoli is perhaps a result of the physiological condition and

not directly related, either as cause or effect, to the enlargement of the nuclei. In cells of the synergium wall, where the ratio of nucleus to nucleolus is lowest, the nuclear structure, as previously mentioned, is characteristic. Expressing it briefly, we may say that the size of the nucleus and of the nucleolus are interdependent, but that the ratio they bear to one another may vary with physiological conditions.

#### *Mitosis in the Sporophyte.*

As the spireme becomes defined in early prophase, the nucleus and the nucleolus enlarge somewhat (Table III). At the same time the nucleoli are often drawn out to a point and connected with the spireme (Plate 26, fig. 5c). As the chromosomes become arranged on the equatorial plate the nucleoli lose some of their recently acquired size—26 of them at metaphase having an average diameter of 2.6 microns. In Regaud and in F.W.A. material the internal structure disappears during prophase, and metaphase nucleoli stain densely all over (Plate 26, fig. 7d). During metaphase the nucleoli are very frequently found attached to the ends of chromosomes (Plate 26, fig. 2). Digby found this in *Galtoma* and says the nucleolus is pushed away from the equatorial plate by a chromosome. In *Tmesipteris* appearances suggest rather that the nucleoli are drawn off from the ends of chromosomes (Plate 26, fig. 2). It is the rule for nucleoli to remain visible during mitosis of *Tmesipteris*, and only in one of some hundreds of mitotic figures examined were nucleoli lacking at metaphase.

When the chromosome groups begin to move apart, the nucleoli, by this time lying free in the cytoplasm, are generally arranged between the chromosome groups and the poles. In connection with the grouping of the nucleoli, some around each chromosome group, there arise two questions—what is the total number of nucleoli present at this time (anaphase) and whether they are partitioned between daughter nuclei in a definite manner or by chance? The first of these questions is difficult to answer, for the deeply-staining chromosomes are likely to conceal some nucleoli, the result being a count that is rather low. In Table IV are given the numbers and manner of distribution of the nucleoli in 57 nuclei in late metaphase, anaphase and early telophase. These nuclei were taken at random and no selection of any kind was exercised. Where the allocation of one nucleolus at metaphase or anaphase is not certain, this is indicated by bracketing that nucleolus.



Table IV.—Nucleoli at Mitosis

Total No. of Nucleoli in Mitotic figure	No. of Cells in which found.	No. of Nucleoli in daughter cells	No. of occurrences of such distribution
7	1	4 3	1
6	8	$\left\{ \begin{array}{l} 3 (1) 2 \\ 3 3 \\ 4 2 \\ 5 1 \\ 4 (1) 1 \end{array} \right.$	3 1 2 1 1
5	33	$\left\{ \begin{array}{l} 3 2 \\ 4 1 \end{array} \right.$	28 5
4	11	$\left\{ \begin{array}{l} 2 2 \\ 3 1 \end{array} \right.$	5 6
3	4	2 1	4

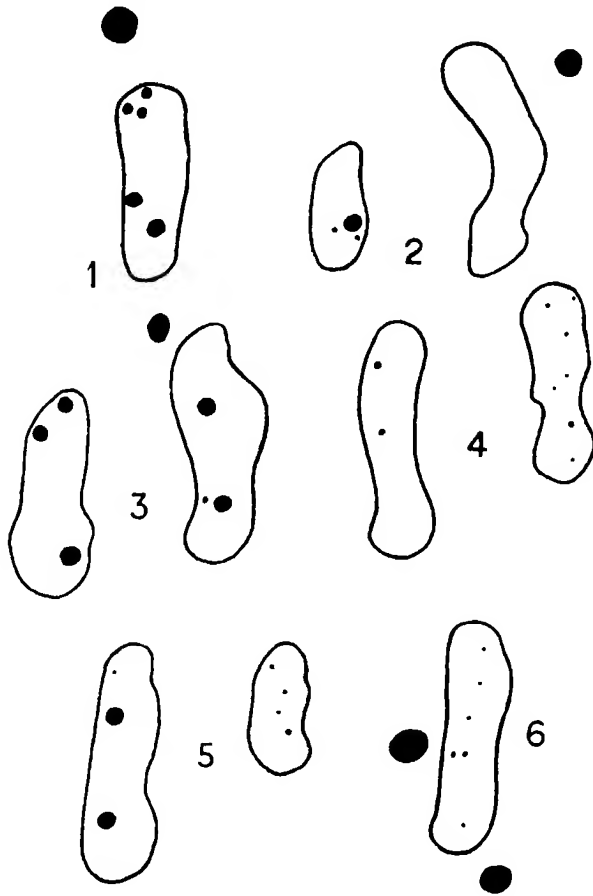
The total number of nucleoli in 57 dividing nuclei was 276—an average of 4.84 per nucleus. Comparing this with the average of 5.06 per nucleus in 62 resting nuclei, it will be seen that the difference (4 per cent.) is no greater than we should expect to find as a result of the obscuring of some nucleoli by the chromosomes. The predominance of the number 5 in Table IV, as in Table I, also shows that no important change in number can occur at mitosis. The presence of seven nucleoli in a single figure is most uncommon, and this is the only occurrence of such a number in the material examined. In one mitotic figure a nucleolus being drawn off from a chromosome was becoming abstricted across the middle, no doubt on account of the tension. The seventh nucleolus in the above figure may well have arisen in the same manner. Occasional fusions of nucleoli lying on the spindle have been observed.

The distribution of nucleoli to sister cells seems, from Table IV, to follow no definite rule.

#### *The Nucleolus at Telophase*

When the telophase nuclei lose the opacity of the anaphase groups, several nucleoli can be seen already formed (Plate 26, fig 4). There are generally present at early telophase about six main nucleoli, and a varying number of smaller bodies in the process of fusion with the main nucleoli. The subsidiary bodies, more rarely the main ones, are often continuous with the telophase chromosomes, suggesting a transference of material from the latter to the nucleoli. As a rule the nucleoli are fairly completely developed when they first become visible, but in some corrosive-acetic, methyl blue-acid fuchsin material

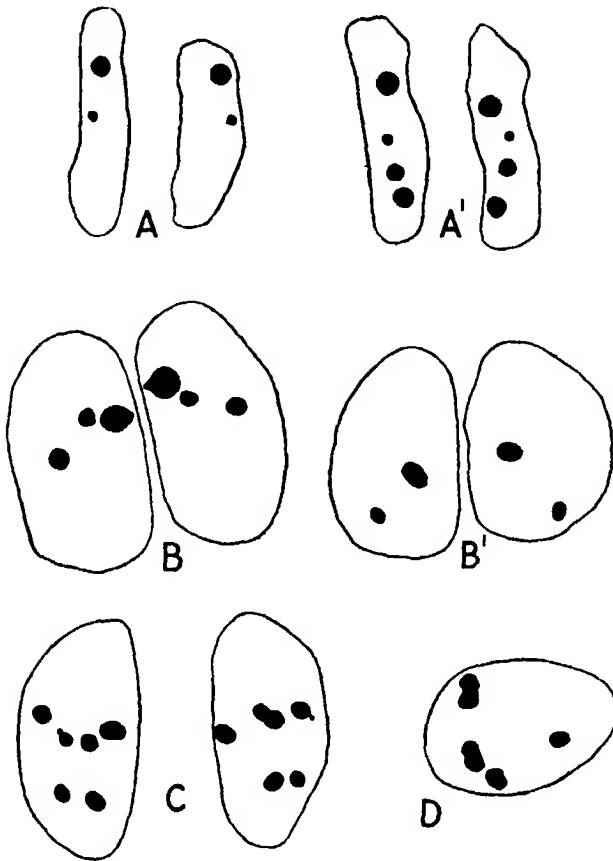
earlier stages could be seen (text-fig. 1). In addition to the full complement of main nucleoli, several small ones of varying sizes were seen in many nuclei



Text-figure 1—Series of six sections of two sister telophase nuclei, showing numerous nucleoli inside, and five still present outside (Corrosive-acetic methyl blue-and fuchsin.)

The fixation of this piece of material was certainly abnormal, but the nucleoli of resting cells were quite normal, disposing of any suggestion that the high number in telophase cells was due to fission. The reason for the greater ease of observation appears to be that the chromosomes stained with methyl blue in a most diffuse manner. The nucleoli were unmistakably red. The presence of nucleoli in numbers lower than six per nucleus is merely a continuation of this process of fusion. In text-fig. 2, D, is shown in outline a complete telophase nucleus in polar view. Six nucleoli are here giving rise to a total of four, by the fusion of two pairs of them.

Very frequently it is observed that the nucleoli of sister telophase nuclei correspond very closely in number, in size and in position. Three instances



Text-figure 2.-- Sections of three pairs of telophase of telophase nuclei, A, A' ; B, B' , C. Note similar arrangement of nucleoli in members of each pair. At D is a complete telophase nucleus in polar view. Two fusions of nucleoli are occurring in it.

are given in text-fig. 2 (A, A' , B, B' ; C). In view of the relative positions of nucleoli at telophase (text-fig. 2) and of the relationship between chromosomes and nucleoli at telophase (Pl. 26, fig. 4) and at metaphase (Plate 26, fig. 2), it seems highly probable that the nucleoli arise at telophase from certain chromosomes which supply the bulk of the nucleolar material. The sister halves of any one chromosome will tend to remain opposite one another in telophase nuclei, especially where the number of chromosomes is so high (200) and the plate so broad as it is in *Tmesipterus*. This would account for the greater

frequency of the similar arrangement of nucleoli in nuclei of the type A, A' as compared with the rounded type of nucleus B, B'.

The origin of nucleoli from telophase chromosomes is indicated also by staining tests. If an excess of acid-fuchsin be used in the methyl blue-acid fuchsin mixture, the dense chromosome groups at late anaphase stain red rather than blue. As the nucleoli and telophase chromosomes become defined, they stain in the typical manner—nucleoli red, chromosomes blue. In other words, during a period when no nucleoli are present, the staining reaction of the chromosomes changes, and when the nucleoli are formed this peculiarity of staining becomes localised in them.

Reverting now to late anaphase, it must be decided what happens to the nucleoli cast out of the original nucleus at metaphase. They are still present during anaphase, without losing anything in size as compared with metaphase nuclei. From anaphase onwards they disappear, as a rule without any signs of fragmentation or degeneration. In Bouin or F.W.A. material, stained in iron-haematoxylin, the nucleoli may remain for some time as unstaining, refractive bodies. More rarely they become smaller and irregular and finally become indistinguishable from the remainder of the cytoplasm. I have so far been quite unable to find any evidence that these nucleoli give rise to chondriosomes when they disappear.

At the time when nucleoli are visible in the telophase nuclei, the nucleoli from the last cell-generation may still be present in the cytoplasm. Since they are disappearing at this time, a series of counts like those made in resting nuclei and at anaphase would have little significance. Several instances have been seen where five nucleoli were outside of the telophase nuclei (text-fig. 1), and in two cases six nucleoli were present in the cytoplasm. It is obvious that in these nuclei the nucleoli must have arisen *de novo*.

#### *Meiosis in Tmesipteris.*

The following account of meiosis is in no way a study of chromosome behaviour. Its object is to follow the behaviour of the nucleoli during this stage, a point of some interest since the prothallus has a reduced number of nucleoli.

Leptotene nuclei contain a very fine, lightly staining chromatin thread and the usual number of nucleoli. As synapsis approaches the nucleoli fuse to form one or two larger bodies. The nucleoli are quite homogeneous after all the fixatives used. Buds are now freely abstracted from the nucleoli and dispersed throughout the nucleus, where they appear to be dissolved (Plate 26, figs. 6b, 6c). Meanwhile the chromatin-thread, still very fine, though staining

more deeply, contracts to one side of the nucleus, which increases from about 23 microns to 35 microns in diameter. Plate 26, fig. 3, represents the stage taken to be the transition from synapsis to second contraction. The chromatin thread has become much coarser, but still stains very lightly. The nucleolus still buds off fragments. In F W A. material the nucleolus has generally the appearance shown in Plate 26, fig. 3. The "buds" arise only from the deeply-staining portions. That the lighter part of the nucleolus does not correspond to the vacuoles in other nucleoli is shown by the fact that this part frequently contains vacuoles. The nucleolus in material fixed in the acetic-acid mixtures is typically vacuolated at this stage.

The coarse thread shown in fig. 3 comes to fill the nucleus and gains in staining power. At the commencement of second contraction the outline of the thread becomes irregular, though distinct chromomeres are not visible. As this gives rise to the dense knot of second contraction, loops project across the nuclear cavity. The limbs of these loops often show a tendency to lie parallel, and the "chromomeres" on such parallel limbs correspond. This is unmistakable, since the "chromomeres" in each thread are irregular in size. No signs of parallel threads were seen at any other stage. Coming out of second contraction, the chromosomes extend until they fill the nuclear cavity with a delicate network of lightly-staining threads, in which are seen one or two much-vacuolated nucleoli. The bivalents arise by condensation on portions of this thread.

The number of bivalents on the equatorial plate is very high, and crowding makes them difficult to count. Six plates carefully outlined under camera lucida ( $\times 3000$ ) gave the numbers: 90, 96, 101, 104, 94, 93— an average of 97. The plates with 101 and 104 were the best for counting, so that an estimate of 100 bivalents seems reasonable. The nucleoli pass either to the same pole or to opposite ones (Plate 26, fig. 9), and are seen outside of the "resting" nuclei formed previously to the homotype division. These nuclei do not reach a stage of complete rest, so that their nucleoli are difficult to count. The usual number appears to be three, though a fourth was occasionally seen—in some cases as if fusing with one of the others. On the heterotype spindle the maximum number counted was three (Plate 26, fig. 8), with the exception of a single spindle which had four. These nucleoli also remain in the cytoplasm after the nuclear membrane has been formed. The spore-nuclei are very favourable for the counting of nucleoli. Of 50 uncut spore-nuclei, 27 had three nucleoli each, 20 had two each, and three had each a single nucleolus.

*The Nucleoli of the Tmesipteris Gametophyte*

The close relationship between nucleoli and chromosomes in the sporophyte suggested that the number of nucleoli might be reduced in the haploid generation.

Twenty-one uncut nuclei near the growing-point of the gametophyte were examined carefully. Of these, 15 had two nucleoli each, five had three each, while one had a single nucleolus. In addition, scores of gametophyte nuclei have been examined to see that none had a number exceeding three. Four egg-cells examined had three nucleoli each, and three more had two each. Taking into account also the counts from 50 spores, given above, it seems plain that three is the maximum number of nucleoli found in resting nuclei of the gametophyte. Comparing this with the maximum of six in the sporophyte, one cannot but be struck by the analogy with chromosome reduction.

That the lower number of nucleoli is due to the smaller size of the gametophyte nuclei is improbable for several reasons. First, Table I shows that in the sporophyte the number of nucleoli is independent of the size of the nucleus, in spite of differences in size greater than the difference between sporophyte and gametophyte nuclei (see Table II).

Secondly, although the egg-nuclei are fully the size of sporophyte nuclei, they still have the reduced number of nucleoli. Thirdly, excepting the egg-nuclei on the grounds that they are old and shrunken, then the relative sizes of nucleus and of nucleolus in similar regions (*p q*, behind growing point) of sporophyte and gametophyte, are practically the same. (See Table III.)

During mitosis the nucleoli of the gametophyte behave as do those of the sporophyte. In two instances of mitosis seen, two nucleoli were at one pole, none at the other. A whole antheridium was seen in mitosis, but the confusion of mitotic figures and of nucleoli prevented any satisfactory counting.

## DISCUSSION.

*The Continuity or Discontinuity of Nucleoli.*

Carleton (14) has shown that even where nucleoli seem to disappear in prophase of mitosis, there may remain a core not shown by ordinary technique, which is represented in each of the daughter nuclei. It has been suggested by Carleton (*loc. cit.*) and by Gatenby (15) that by such means the nucleolus may perhaps be an independent, self-perpetuating body. Schaffner (22) holds similar views for *Lilium*. On the other hand, Gates (8, 9, 10), and Mottier, hold

that in cases described by them, nucleoli arise in daughter cells *de novo*, the old nucleoli remaining in the cytoplasm. The phenomena in *Tmesipterus* strongly support this view, for in this plant the number of nucleoli is fairly definite, and the complete number may be seen in the cytoplasm after a new generation of nucleoli is formed in the telophase nucleus.

#### *The Dependence of Nucleoli on Chromosomes.*

The close relationship between nucleoli and chromosomes is not favourable to the view of genetic continuity of nucleoli. This relationship is shown in several ways :-

(a) *Sex-chromosomes and nucleoli* - Stevens (*loc. cit.*) describes the sex-chromosomes of 21 species of *Coleoptera*. Only in seven of these species is a nucleolus described in spireme or later stages of meiosis, and in every one of these seven species the nucleolus is regularly attached to the sex-chromosomes, sometimes as late as diakinesis. Agar (18) and Greenwood (19) have found exactly similar conditions in the three genera of marsupials described by them. Agar (*loc. cit.*), in *Petaurordes*, finds that the plasmosome of secondary spermatocytes differs according as the X chromosome or the Y chromosome is present.

(b) *The Origin of Nucleoli at Telophase* - The initiation of nucleoli at telophase by the flowing together of smaller bodies, usually in relation to chromosomes, has been described by several authors in different plants. These include *Galtonia* (Digby, 1), *Vicia faba* (Fraser and Snell, 23), *Allium Cepa* (Reed, 3), *Polytrichum formosum* (Walker, 24), *Munum hornum* (Wilson, 25). It has already been shown that the same thing occurs in telophase of *Tmesipterus* and that the relationship of nucleoli to certain chromosomes is indicated by the corresponding positions of nucleoli in sister-nuclei.

(c) As a corollary to the above, we should expect that high chromosome numbers should be associated with greater numbers of nucleoli. This expectation is fully borne out in the cases given below, taken indiscriminately from those available (see Table V).

(d) Nucleoli are frequently in direct connection with the prophase chromosomes, and when they persist are sometimes intimately connected to metaphase chromosomes—*Galtonia* (Digby, 1); *Tmesipterus*.

#### *The Relationship of Nucleoli to the Chromatin Organisation of the Nucleus.*

The present position with regard to the theory of individuality of the chromosomes and the bearing on it of our knowledge of the nucleolus has been ably summarised by Gatenby (26) in these words: "Either the nucleolus represents

Table V.

Plant or Animal	Observer	Number of Chromosomes	Number of Nucleoli
<i>Equisetum arvense</i>	Beer (28)	115	3-6
<i>Allium Cepa</i>	Reed (3)	16	* (1)-2
<i>Allium triococcum</i>	Nothnagel (29)	16	2
<i>Laboulbenia chrtophora</i>	Faull (30)	8	1
<i>Smilacina racemosa</i>	Woollery (31)	44	1-4
<i>Acer Negundo</i>	Mottier (11)	24	1
<i>Cladophora glomerata</i>	Carter (32)	Too many to count	3-5
<i>Podophyllum peltatum</i>	Mottier (33)	16	" 1 or more "
<i>Tmesipteris tannensis</i>	Present paper	200	3-6
" (haploid)	"	100	1-3
<i>Thyrium filix foemina</i>	Fatmer and Digby	76-80	1-2
" <i>clariss</i> Bolton	" " (34)	84	" A number "
" <i>clariss</i> Jones	" "	90	" More abundant "
" <i>conglomeratum</i>	" "	100	" Also numerous "
<i>Trirhabda virgata</i>	Stevens	28	1
" <i>canadense</i>	"	28	1
<i>Odontota dorsalis</i>	" (16) (17)	16	1
<i>Epilachna borealis</i>	"	18	1
<i>Euphorbia unda</i>	"	20	1
<i>Blepharida rhous</i>	"	32	1
<i>Nilpha americana</i>	"	40	* (2)-3
<i>Termopsis angusticollis</i>	"	52	2
<i>Stenopelmatus</i>	"	47	3
<i>Blattella germanica</i>	"	23	1
<i>Ipais cenothea</i>	"	10	1
<i>Phaseolus arctus</i>	(Greenwood (19)	16	* (1)-2
<i>Macropus</i>	Agar (18)	12	1
<i>Petauroides</i>	Agar (18)	22	(1) (?) -2

\* Indicates that the author of the original paper regards higher number as arising by fission from the bracketed number.

a second chromatin of some kind, but separate from the chromosomes, or it derives its chromatin from the chromosomes, or there is some cell-substance other than chromatin which has the attribute of forming bodies similar to the ordinary nuclei except for the presence in them of true chromatin." This statement is based chiefly on the origin of secondary nuclei from the nucleolus of plants (Gates, 27) and of animals (Gatenby, 12) (Hogben, 13). Other phenomena such as the emission of "chromidia" from the nucleolus or from the nuclear-reticulum, and the manner in which chromosomes at prophase sometimes develop at the expense of the nucleolus, render it highly probable that the nucleolus does derive its "chromatin" from the chromosomes and that much of it may be lost. Doncaster ('Cytology,' p. 198) suggests that these facts may be reconciled with the theory of individuality of the chromosomes, by assuming that the persistent parts of chromosomes are mere frameworks



on which varying amounts of chromatin may be concentrated at different times.

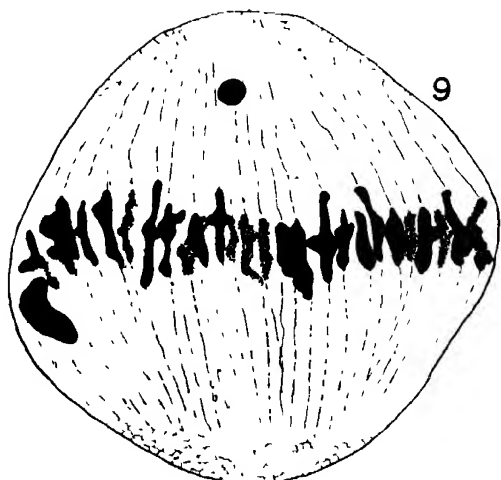
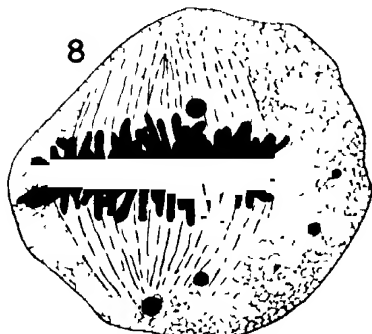
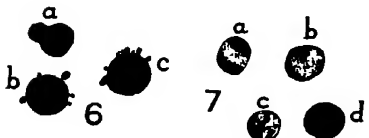
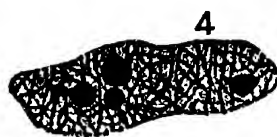
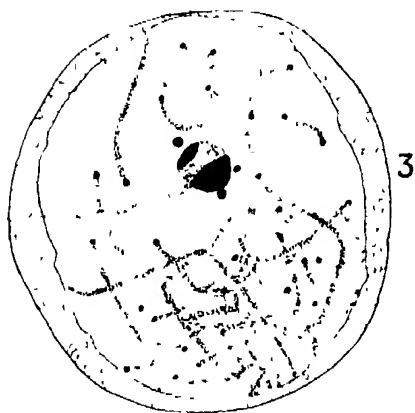
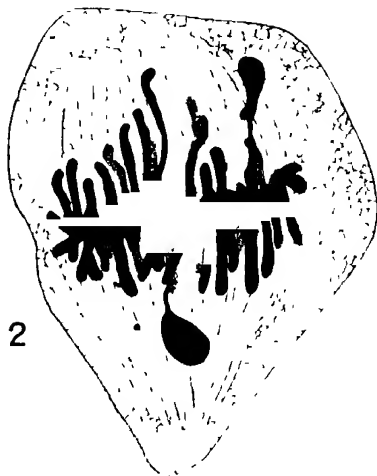
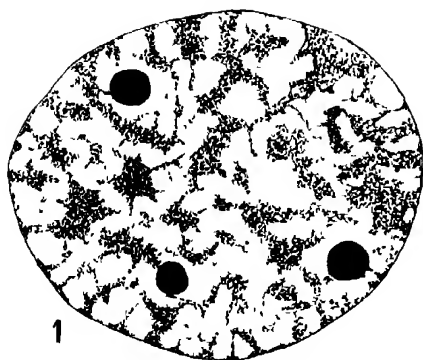
As to the possible function of the nucleolus, it is clear that theories such as chromosome-building, or spindle forming, fail in general application when we consider such cases as *Tmesipteris*. Their origin from chromosomes in both plants and animals, and the irregular manner of their allocation during mitosis in *Tmesipteris*, suggest rather that nucleoli are products of the metabolism of chromosomes in the compact, deeply-staining form, and that their subsequent fate depends on the metabolism of the particular organism in which they arise. The frequent association of plasmosomes and sex-chromosomes is in accord with this view, for sex-chromosomes are characteristically in the compact state over a much longer period than are the autosomes in the same species.

#### CONCLUSIONS.

1. The maximum number of nucleoli seen in resting nuclei of *Tmesipteris* is six for the sporophyte, three for the gametophyte. Numbers lower than these are produced by fusion of nucleoli.
2. At telophase of mitosis the nucleoli arise *de novo* by the fusion of a number of droplets of nucleolar material.
3. The nucleoli of sister-cells often arise in corresponding positions, numbers and sizes.
4. At prophase the nucleoli are connected with the spireme and increase in size as the spireme becomes defined.
5. At metaphase the nucleoli become detached from the chromosomes and pass some to each daughter cell.
6. The distribution of nucleoli to daughter-cells follows no definite rule, nor do these nucleoli re-enter a nucleus.
7. At synapsis the number of nucleoli is reduced by fusion to one or two, which bud freely.
8. Both at the heterotype and at the homotype mitosis the nucleoli persist as in the vegetative mitosis.
9. The reduction from "diploid" to "haploid" number of nucleoli coincides in point of time with chromosome reduction.
10. The chromosome number (diploid) of *Tmesipteris* is approximately 200.
11. The relationship of nucleoli to chromosomes is briefly discussed.

In concluding, I should like to express my deep gratitude to Professor H. B. Kirk for his untiring aid and encouragement, to Dr. L. Cockayne, F.R.S., for





help he has freely given, and to Mr. A. W. Waterworth who lent a valuable microscope objective.

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## DESCRIPTION OF PLATE 26.

*All figures*  $\times 1700$ .

FIG. 1.—Section of resting nucleus of *Tmesipteris synangium* (corrosive-acetic).

FIG. 2.—Mitosis, showing nucleoli in connection with chromosomes (Bouin).

FIG. 3.—Stage between first and second contractions. The nucleolus is budding (F.W.A.).

FIG. 4.—Telophase of mitosis. Nucleoli appear to be continuous with chromosomes. (Bouin.)

FIG. 5.—Nucleoli, A. and B. from resting nucleus, C. from prophase, D. from anaphase. (Bouin.)

FIG. 6.—Nucleoli, A. from leptotene (corrosive-acetic), B. and C. from synapsis (F.W.A.)

FIG. 7.—Nucleoli, A., B. and C. from resting archesporial cells, D. from metaphase of mitosis. (Regaud)

FIG. 8.—Homotype mitosis. The full complement of three nucleoli is seen. The two on right are at the pole of another spindle (Bouin.)

FIG. 9.—Heterotype mitosis. Two nucleoli are present (Bouin-methyl blue-acid fuchsin.)

### *Investigation on the Crystalline Lens.*

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Although a large number of papers have been written on the nature and possible causes of cataract, it is only recently that any attempt has been made to attack the problem from a biochemical standpoint. It was thought, therefore, that experiments on the biochemistry of the normal animal lens might be of some value in the solving of this problem.

Since the experiments in this paper are concerned chiefly with the nature of the autoxidation system in the lens, a brief account may be given of the facts discovered by previous workers on this subject.

It was found by Arnold (1910) that fresh animal tissues, such as muscle, give a strong purple coloration with a mixture of sodium nitroprusside and ammonium hydrate. In 1912, Reis showed that a fresh human lens gives a marked positive reaction to the same test, whereas cataractous lenses which he examined gave only partial reactions, *e.g.* the nucleus alone being positive, or they were wholly negative. In 1920, Jess separated the three chief proteins of the lens, after the manner originally devised by Morner (1895), and found that one protein,  $\beta$ -krystallin, is especially rich in cystein, and gives a markedly positive nitroprusside reaction. He also found that in cataractous lenses in which the nitroprusside reaction was negative, the water-soluble  $\beta$ -krystallin apparently had been replaced by the insoluble protein albumoid. In the case of other

tissues a positive nitroprusside reaction was said to indicate an active metabolism (Arnold, 1910), and the work of Reis and Jess showed that this fact was probably also true of the lens. Goldschmidt has indeed recently shown (1924) that the lens contains a balanced autoxidation system of the type which Hopkins discovered in other tissues, such as muscle (1922), *i.e.* a balanced oxidation-reduction system between a soluble constituent, *viz.* glutathione, and a thermostable protein residue. Both of these constituents give a positive nitroprusside reaction, and Goldschmidt found that either or both of them might be absent from a cataractous lens. An autoxidation system of this kind might reasonably be expected to be of special importance in the metabolism of a non-vascular tissue such as the lens, wherein no rapid oxygenation could occur.

In these experiments I have also made a preliminary attempt to discover any possible effect of heat rays or ultra-violet light on the autoxidation system of the lens, for both these agents have been cited as causes of cataract.

#### METHODS.

Fresh ox and sheep lenses were used, and in all the experiments on the oxygen uptake of the lens, parallel results were obtained by two methods.---

A. Thunberg's methylene blue-technique.

B. Direct measurement of the oxygen uptake in a Barcroft micro-respirometer.

In each experiment of either type, the lens was suspended whole, or finely ground, in aqueous humour, or in a medium of the same alkalinity (*pH* 7.6), such as Mammahan Ringer, or phosphate or borate buffer. All experiments were performed in a water-bath at 35° C.  $\pm 0.01^\circ$ .

*Method A.* In an experiment of this type, strictly comparable amounts of lens tissue, and of the appropriate solutions, together with equal volumes of methylene-blue solution), were introduced into vacuum tubes of equal size. They were evacuated simultaneously until the contained liquids boiled. The taps were then turned, and the tubes were placed in a water-bath at 35° C. The time taken by each tube to decolorise was noted.

*Method B.*—In the direct method, two types of Barcroft respirometers were used: (*a*) For measuring the oxygen uptake of a whole lens, the older type of bottles with round bottoms were found to be most suitable. In them a whole

sheep's lens could be kept covered by 2 c.c. of fluid. (b) For suspensions of freshly ground or dried lens tissue, flat-bottomed bottles of the type generally used for tissue-respiration experiments were most convenient. In both types of bottles there were small cups of potash for the absorption of  $\text{CO}_2$ . In performing comparative and control experiments, care was taken that the apparatus used was of the same type, that it was immersed in the same water-bath, and shaken at the same rate. In each apparatus, the reacting mixture was introduced into the right-hand bottle, while the left one contained an equal volume of distilled water. Each bottle was connected by a three-way tap with one arm of the manometer, which registered the oxygen uptake.

#### REMOVAL OF THE LENS FROM THE EYE

The sheep and ox eyes were brought on ice fresh from the slaughter-house. The cornea of each eye was wiped dry and clean, and then punctured with a hot needle to allow the aqueous humour to escape. It was then an easy matter to open the cornea by a cross-shaped incision, without damaging the underlying lens. By the application of gentle pressure to the eyeball, the lens could be brought forward through the pupil. The capsule was then ruptured by the tip of a needle, and the lens was received on to a watch-glass, uninjured, and free from contamination by other tissues in the eye. When required, it was weighed between two watch-glasses.

A clear suspension of lens tissue was made by grinding the fresh lens with a known volume of water, buffer, or Ringer. Dried lens was prepared by grinding the fresh lens alone in a glass mortar. The foamy mass would dry if left in a vacuum desiccator overnight, and it could then be ground to a fine white powder.

Glutathione was used in the form of the hydrochloride, neutralised, and dissolved in the appropriate buffer solution - 5 mgm. glutathione hydrochloride in 1 c.c. solution. Linseed oil was used in a freshly made aqueous suspension of 5 mgm. oil in each 1 c.c. Acids, *e.g.* malic, succinic and fumaric, were also made up in buffer solutions of 5 mgm. acid to each c.c. solution. Phenyl urethane was used as an antiseptic in all experiments by the direct method.

#### EXPERIMENTAL DATA.

Throughout the experiments the nitroprusside reaction of the lens was used as a guide to the chemical changes which occurred. In testing, a mixture of

crystalline ammonium sulphate, sodium nitroprusside, and of ammonium hydrate was employed as suggested by Hopkins.

### 1 Oxygen Uptake of Lens.

(i) A crystalline lens has a small but definite oxygen uptake. The type of curve obtained from an experiment by the direct method A is distinct in shape (see fig 1) and shows a fairly rapid preliminary uptake, followed by a

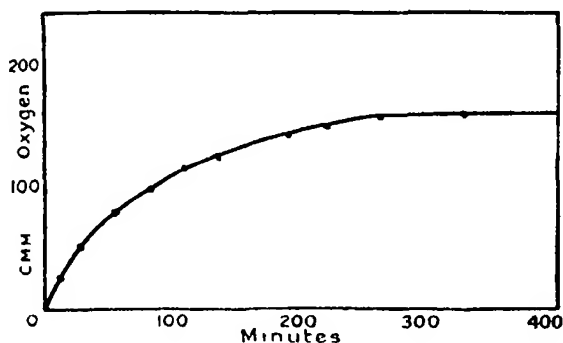


FIG 1 —(Experiment 9 ) Oxygen uptake of whole Ox Lens. (Wt. 1.964 gm in 2 c.c. Ringer ) Phenyl Urthane present.

gradual increase. In two hours an ox lens, about 2 gm. in weight, will use, on an average, 125-130 c mm. of oxygen. (ii) A dried lens has a very much smaller uptake, and reduces methylene blue extremely slowly. (iii) Dialysis also renders the oxygen uptake practically nil, owing to the removal of glutathione. (iv) A suspension of finely ground fresh lens tissue gives the same type of uptake curve as the whole lens, but in comparison with the latter it has a more rapid oxygen consumption. (v) A suspension of dialysed lens will not reduce methylene blue at all —Cf Fresh lens tissue will reduce methylene blue rapidly, and if air be readmitted into the tube at the end of the experiment, reduction will again occur. This is an indication that the autoxidation system is reversible.

### 2. Effect of Glutathione and Linseed Oil on the Lens,

(Note.- Experiments were made with linseed oil in particular, because Hopkins, Meyerhof, and others have shown that it has a remarkable catalytic effect on the autoxidation system of other tissues.)



(i) Reference to fig. 2 shows that the oxygen uptake of a fresh lens is

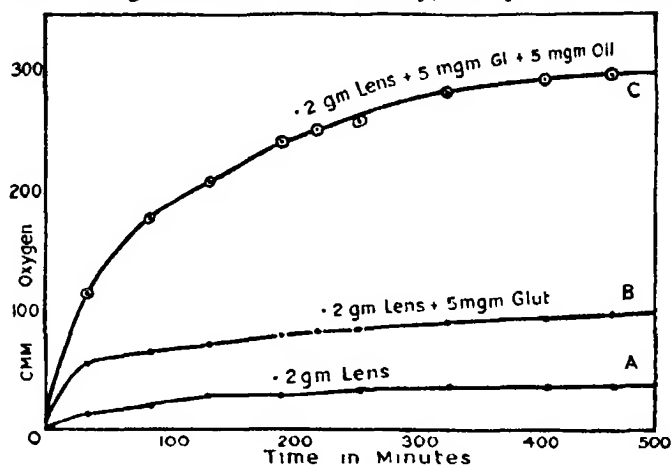


FIG. 2 —(Experiment 23.) A Oxygen uptake Lens alone. B. Oxygen uptake Lens + Glutathione. C. Oxygen uptake Lens + Glutathione + Linseed Oil. (Borate buffer pH 7.6)

increased in the presence of glutathione, and markedly so if linseed oil is added as well.

(ii) Addition of glutathione to a dialysed suspension of lens tissue restores its power to reduce methylene blue, and its oxygen uptake also (*cf.* the effect of glutathione on well-washed muscle tissue).

(iii) Fig. 3 shows the effect of glutathione on dried lens tissue. It is interesting to note that the curve is of the typical shape.

(iv) The following experiment (Table I) may be compared with the effects shown in fig 2 :—

Table I.—Experiment on the Effect of Glutathione on Freshly Ground Ox Lens.

N.B.—Each tube contains { 0.05 c.c. M/1,000 methylene-blue solution.  
2 c.c. Ringer

Temperature of bath, 35° C.

Lens tissue and glutathione each suspended in Ringer.

		Reduction time.
Exp. 1.—Tube A.	0.4 gm. lens alone	150 mins. }
„ B.	0.4 gm. lens, 5 mgm. glutathione	50 „ }
Exp. 2 — „ A.	0.5 gm. lens alone	114 mins. }
„ B.	0.5 gm. lens, 5 mgm. glutathione	42 „ }
Exp. 3. „ A.	0.8 gm. lens alone	75 mins. }
„ B.	0.8 gm. lens, 5 mgm. glutathione	30 „ }

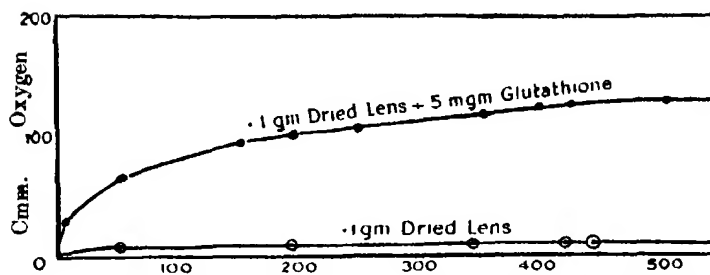


FIG. 3. (Experiment 4) Effect of Glutathione on Dried Lens. (In water buffered to 7.6)

### 3 The Thermostable Residue in the Lens.

Goldschmidt has recently shown (March, 1924) that the non-dialysable protein residue of the lens will function as a thermostable residue (*i.e.* it reacts with oxidised glutathione or cystine and reduces these substances; in the living tissue the reaction is probably a reversible one). He used only the methylene blue technique, so that the results here obtained by the direct method serve to confirm his observations

A quantity of thermostable residue was prepared in the following manner: - About 50 fresh sheep lenses were ground finely with N/10  $\text{H}_2\text{SO}_4$ , and then boiled with the acid. The precipitated proteins were then removed by filtration, and the residue was reground several times with distilled water until the filtrate no longer gave a nitroprusside reaction. It was then dried with alcohol, and *in vacuo* over sulphuric. When dry, it was ground to a fine white powder. In order to render it free from lipoids, it was subjected to three days' extraction in a Soxhlet continuous extractor with alcohol and ether in turn. The specimen so obtained gave a positive nitroprusside reaction and was insoluble in water

(i) The thermostable residue, when tested, had no power to reduce methylene blue, but did so readily in the presence of a small amount of glutathione.

(ii) By the direct method it was found that a lipid-free specimen of T.S.R.\* has no oxygen uptake. With a few milligrammes of glutathione a curve was obtained, showing a definite consumption of oxygen, which reached its maximum in  $2\frac{1}{2}$  hours (*see fig 4*). At the end of the reaction the nitroprusside test was negative. (In comparison, the curve given by the fresh lens showed a slowly progressive uptake, and the nitroprusside reaction was never lost)

(iii) Addition of linseed oil as well as glutathione, caused a still more marked increase in the oxygen uptake

\* T.S.R. = Thermostable Residue.

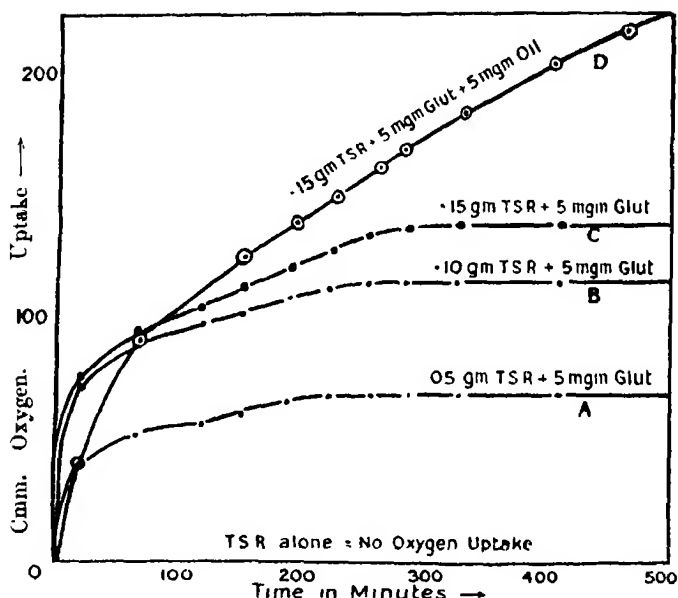


FIG. 4.—(Experiment 43.) A. 0.05 gm. T.S.R. + 5 mgm. Glutathione. B. 0.10 gm. T.S.R. + 5 mgm. Glutathione. C. 0.15 gm. T.S.R. + 5 mgm. Glutathione. D. 0.15 gm. T.S.R. + 5 mgm. Glutathione + 5 mgm. Linseed Oil. (Borate buffer, pH 7.6.) Behaviour of Lipoid-free Thermostable Residue.

#### 4. The Nature of the Thermostable Residue.

Mörner (1894) separated and identified three proteins in the lens, viz., two water-soluble proteins,  $\alpha$ -krystallin,  $\beta$ -krystallin; and an insoluble albumoid. Of these Jess showed (1920) that  $\beta$ -krystallin gives a strong positive nitroprusside reaction, whereas  $\alpha$ -krystallin is only weakly positive, and albumoid is negative. In view of these facts I considered it possible that one protein, viz.,  $\beta$ -krystallin, might act as a thermostable residue more than the others.

Specimens of the three proteins were prepared as follows, by a slight modification of Mörner's method:—

A large number of sheep lenses were ground with a large volume of quarter-saturated sodium chloride solution. The solution was shaken for at least an hour, and was then left overnight in the ice chest. A small amount of insoluble protein collected at the bottom of the flask, and was separated from the clearer solution (A) by centrifuging.

*Albumoid.*—The insoluble residue so obtained was again treated with NaCl as before, and the process was repeated until the decanted clear solution gave no test for protein. The residue was then washed by grinding with distilled

water, and was finally centrifuged, and dried with alcohol and *in vacuo* over sulphuric. This specimen of albumoid gave no N.P.R.\*

*$\alpha$ -krystallin.* After dialysis in a collodion sac the solution (A) (containing the water-soluble proteins) was treated with dilute acetic acid until the latter was present in 0.03 per cent. concentration. A fine precipitate of  $\alpha$ -krystallin came down in the cold, and was removed from the clear solution (B) by centrifuging. It was purified by being dissolved in dilute 0.01 per cent ammonia and re-precipitated by 0.01 per cent acetic acid. The pure solution of  $\alpha$ -krystallin was precipitated by alcohol, then centrifuged, and dried *in vacuo*. The final product had a weakly positive N P R

*$\beta$ -krystallin* was obtained from the clear solution (B). The latter after all traces of  $\alpha$ -krystallin had been removed, was dialysed free from acetic acid, and was saturated with crystalline  $\text{MgSO}_4$  at  $30^\circ \text{C}$ . A bulky precipitate was formed which settled in the cold and was easily centrifuged off. It was dissolved in distilled water, dialysed and dried in the way described for  $\alpha$ -krystallin. The specimen had a strong positive N P R

Each protein was obtained as a white powder, and a certain weight was converted into thermostable residue in the manner described for the whole lens. These preparations were used for the following experiments:—

(1) Fig. 5 shows that  *$\beta$ -krystallin* reacts very readily with glutathione and

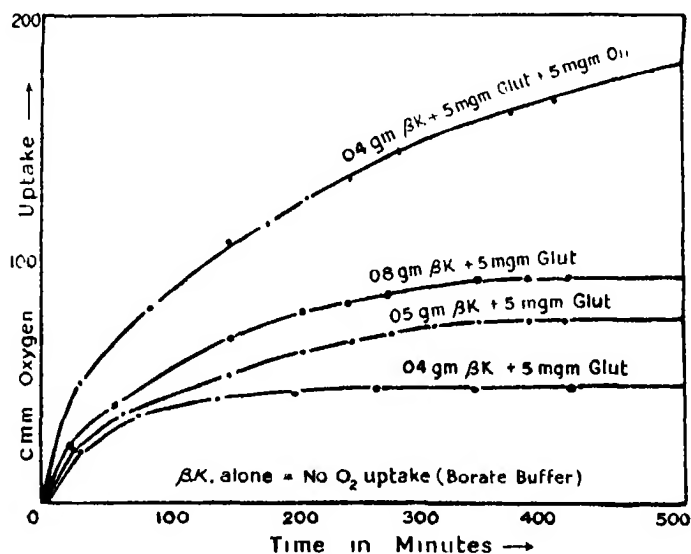


FIG. 5. (Experiment 42.) Action of  $\beta$ -Krystallin as a Thermostable Residue.

\* N.P.R. - Nitro-prusside Reaction.

with glutathione and linseed oil. In fact, the oxygen uptake of a known weight of  $\beta$ -krystallin in the presence of 5 mgms. glutathione closely approximates that of an equal weight of lens T.S.R. under the same conditions (cf. figs. 4 and 5) (Note also that  $\beta$ -krystallin constitutes the bulk of the protein of the lens) At the end of each of these experiments the nitro-prusside reaction had disappeared

(ii)  $\alpha$ -krystallin gave only a very slight reaction with glutathione (see fig. 6),

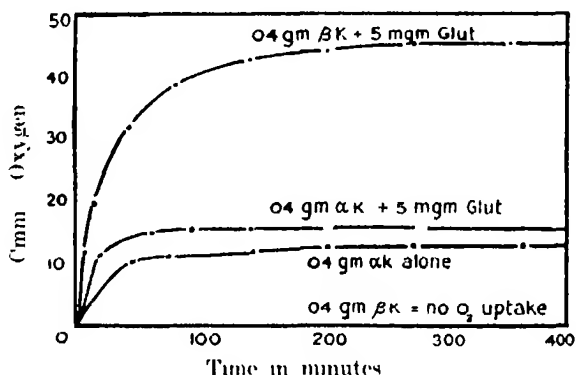


FIG. 6 (Experiment 40) Illustrates the almost Negligible Action of  $\alpha$ K and Glutathione.

and it is possible that this was due to the presence of slight traces of  $\beta$ -krystallin, as it is difficult to separate these two proteins entirely

(iii) A dialysed solution of  $\alpha$ -krystallin showed no reduction of methylene blue after four hours, even when glutathione was present

(iv) *Albumond* did not react at all with glutathione in either the direct or indirect methods of experiment

### 5 The Glutathione Content of the Ox Lens

Tunnicliffe's method for the estimation of glutathione in animal tissues was used. It is accomplished in two stages —

(a) The precipitation of the proteins by trichloroacetic acid.

(b) The direct titration of the filtrate with a standard solution of iodine.

In each experiment the lens was weighed carefully between two watch-glasses immediately after extraction from the eye. It was then ground in a glass mortar with a certain volume (about 30 c.c.) of 10 per cent trichloroacetic acid. The clearer layers of the solution were decanted off and filtered into a Büchner flask. The remaining suspension was ground with a small volume of distilled water, and it was then filtered into the same flask. The residue on

the filter was washed with distilled water to remove the final traces of acid (the last washings should not give a nitroprusside reaction). The whole of the clear filtrate, including the washings, was titrated directly with a standard N/100 solution of iodine. The point at which a drop of the solution no longer gave a nitro-prusside reaction was taken as the end-point.

*E.g.* Experiment 9 -

*Lens A.*—Weight, 2.1812 gms. Trichloroacetic filtrate requires 3.08 c.c. of 0.008 N iodine

*Lens B.* Weight, 2.094 gms. Filtrate requires 2.91 c.c. of 0.008 N. iodine

On the assumption that 1 c.c. N/100 iodine = 2.5 mgs. glutathione, and that practically the whole of the soluble SH compound in the lens is glutathione (as Goldschmidt also concluded) (*N.B.*—Tunncliffe found that in other tissues 98 per cent. is glutathione).

*Lens A* contains 0.2967 gm. per cent., and *Lens B* 0.3058 gm. per cent. glutathione

As the result of a number of experiments, the average glutathione content of the lens is 0.3052 gm. per 100 gms. weight of lens (*cf.* yeast, 0.22 gm. per cent. and rabbit muscle, 0.042 gm. per cent., as found by Tunncliffe).

A pair of lenses from the same animal in each case gave figures in very close agreement, *e.g.* -

Table II

Weight of lens	Glutathione content	
	Total.	Per 100 gms. weight.
Experiment 8. -		
<i>Lens A</i> 2.2432 gms	6.909 mgms	0.308 gm. per cent. }
<i>Lens B</i> 2.2800 „	6.818 „	0.299 „ „ }
Experiment 10		
<i>Lens A.</i> 2.2220 gms	6.821 mgms	0.3056 gm. per cent. }
<i>Lens B.</i> 2.2270 „	6.758 „	0.3034 „ „ }

(a) *Effect of Exposure to Heat*—It was found that exposure of the lens to moderate heat caused an appreciable decrease in its glutathione content.

A pair of lenses from the same animal were used in each experiment. One was suspended whole in 2 c.c. of Ringer in a test-tube, and the latter was placed in a water-bath at 35° C. for two hours. The other was similarly suspended, but was left in the dark at ordinary room temperature for the

same length of time The glutathione content of each lens was then estimated. Table III shows two typical experiments : —

Table III.

	Weight of Lens	Glutathione Content.	Difference
Experiment 8.---			
	<i>Lens A</i> 2·1812 gms (control)	0·2967 gm. per cent.	} 0·0125 gm. per cent.
	<i>Lens B</i> —2 2454 „ (exposed)	0·2842 „ „	
Experiment 11			
	<i>Lens A</i> - 2·0185 gms (control)	0·3226 gm. per cent	} 0 0122 gm. per cent
	<i>Lens B</i> 2·0195 „ (exposed)	0·3104 „ „	

The decrease observed is certainly not a mere difference between two companion lenses (*cf* Table II) It was also proved that the control lens showed no decrease of glutathione by being left in Ringer.

(b) *Effect of Exposure to Ultra-Violet Light* As in the previous experiments, a pair of fresh ox lenses were used. Each one was suspended whole in 2 c c. of mammalian Ringer. The control one was put in the dark at room temperature, while the other was exposed in a quartz tube to the rays from a mercury vapour lamp, at a distance of 35 cm from the external cover of the lamp Exposure could only be continued for half an hour at a time, as the lamp heated up. 5 to 10 minutes was, however, sufficient for cooling The following are typical experiments —

Table IV.

*N.B.*—(a) The exposed lens was given four half-hour exposures with 10 minute intervals, *i.e.* 2 hours' total exposure.

(b) The temperature of the Ringer suspending the lens rose to 35° C. during the exposure.

(c) The average current of the lamp was 1·7 amps., and its voltage was 85

	Weight of Lens.	Glutathione Content	Difference
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Experiment 4. —

	<i>Lens A</i> —2·327 gms. (control)	0·2454 gm. per cent.	} 0·0239 gm. per cent.
	<i>Lens B</i> —2·275 „ (exposed)	0·2215 „ „	

Experiment 5. —

	<i>Lens A</i> —2·1720 gms. (exposed)	0·2777 gm. per cent.	} 0·0247 gm. per cent.
	<i>Lens B</i> —2·2215 „ (control)	0·3024 „ „	

By comparing Table IV with Table III, it is obvious that ultra-violet rays have a more deleterious effect on the lens than have heat rays

In no case did the lens become clouded after two or three hours' exposure to ultra-violet light under the conditions described above.

#### 6. Effect of Ultra-Violet Light on the Autoxidation System of the Lens.

(a) Lens tissue which has been exposed to ultra-violet light has a smaller oxygen consumption than an equal weight of normal unexposed tissue (*see* fig. 7). It also reduces methylene blue more slowly (*see* Table V)

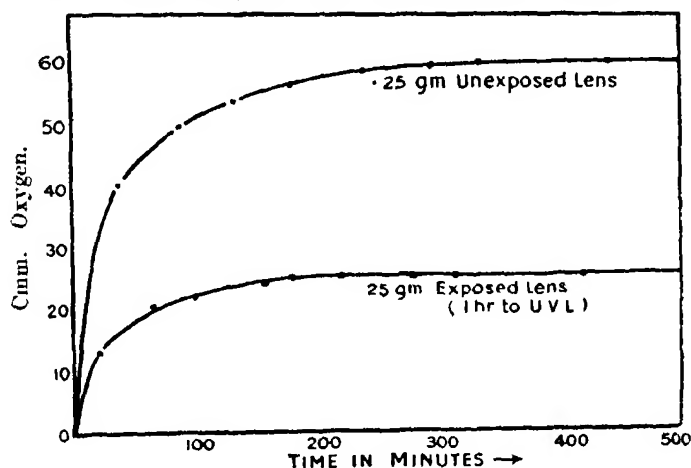


FIG. 7.—Action of Ultra-violet Light on Oxygen Uptake of Lens (Borate buffer 7.6.)

(b) On the addition of equal weights of glutathione to equal weights of exposed and unexposed lens, the rate of reduction of methylene blue was the same in each case (*see* Table V)

Table V

N.B.—In each tube were placed :—

- (a) 0.9 gm of the selected lens tissue—ground and suspended in Ringer (3 c.c.); (b) 0.05 c.c. M/1000 methylene blue solution :

Experiment.—

Reduction Time.

(1) *Lens A.*

(exposed 1 hour) . . . . . { 105 mins. (lens alone)  
30 mins. (lens + 5 mgms. glutathione).

(2) *Lens B.*

(exposed 1 hour) . . . . . { 91 mins. (lens alone).  
32 mins. (lens + 5 mgms. glutathione).

(3) *Lens C.*

(unexposed) . . . . . { 75 mins. (alone).  
30 mins. (lens + 5 mgms. glutathione).



7. *Enzymes in the Normal Lens.*

From methylene-blue experiments it was found :—

(a) The fresh lens can oxidise glucose, fumaric acid, malic acid, and, less readily, succinic acid. (These observations are in agreement with those of Ahlgren 1923) The oxidation was in each case more rapid in phosphate than in borate buffer of the same pH (see Table VI).

Table VI

Experiment 24 —In each tube were placed .—

(a) 0.3 gm. lens tissue ground and suspended in 3 c.c. of either borate, or phosphate buffer of pH 7.7; (b) 0.05 c.c. M/1000 methylene blue solution; (c) 5 mgms. of the required acid or glucose in appropriate buffer

	Reduction Time (Mins.).	
	Phosphate	Borate
(1) Lens alone . . . . .	98	135
(2) Lens + glucose . . . . .	78	98
(3) Lens + fumaric acid . . . . .	60	82
(4) Lens + malic acid . . . . .	65	103
(5) Lens + succinic acid . . . . .	78	120

(b) After exposure to ultra-violet light, the lens was able to oxidise the above acids less readily (see Table VII).

*N B* The lenses (sheep) were exposed whole, suspended in Ringer for one hour. The acids used were neutralised to the same pH as Ringer, 7.6

Table VII.

Experiment 37A *N.B* —Each tube contains—

(a) 0.4 gm. of lens tissue unexposed or exposed, ground and suspended in 3 c.c. Ringer (b) 0.05 c.c. M/1000 solution of methylene blue; (c) 5 mgms. of the required acid

	Reduction Time (Mins.).	
	Unexposed.	Exposed.
(1) Lens + fumaric acid . . . . .	53	110
(2) Lens + malic acid . . . . .	57	81
(3) Lens + succinic acid . . . . .	81	96

## DISCUSSION.

The chief interest of these investigations lies in any possible application which they may have to the causes of cataract.

The first proven fact is that the lens contains an autoxidation system similar to that which Hopkins found in muscle and other tissues. Goldschmidt has recently demonstrated the same fact by the use of the methylene blue technique. The direct measurement of the oxygen uptake of the lens by the micro-respirometer is, however, a graphic method of confirmation.

The autoxidation system of the lens consists of (1) dialysable glutathione, (2) a thermostable protein residue. These constituents are maintained in constant chemical equilibrium by an oxidation-reduction of an  $\text{SH} \longleftrightarrow \text{SS}$  type. The peculiar mode of nutrition in the lens prevents any excessive use of oxygen or rapid elimination of waste products. It is therefore conceivable that the autoxidation system is of special economic importance in the respiration of the lens, and that it is not merely secondary in character as it is in other tissues. In favour of this view that it is of vital importance to the normal condition of the lens are the facts that (a) in cataractous lenses either or both parts of the autoxidation system may be absent (Goldschmidt); (b) the lens in comparison with other tissues has a relatively high glutathione content.

Because of the etiology of cataract, ultra-violet and heat rays have often been cited as causes of that condition. Thus, there has been a tendency to study the final gross defect of protein precipitation rather than the more insidious onset of primary chemical defects. It is obvious that the health of any tissue depends chiefly on the proper functioning of its respiratory system. By analogy, therefore, if the autoxidation system of the lens is disturbed or partially destroyed, the lens itself must suffer. Ultra-violet light and heat rays cause a decrease in the glutathione content of the lens *in vitro*, when the latter is protected by a surrounding layer of Ringer. It is probable that these rays have the same effect on a lens *in vivo*, although it is unfair to deduce too much from experiments on surviving tissue. It is as yet unknown whether the secondary (thermostable) system is affected by the rays *in vivo*. The precipitation of the proteins and the increase of lipoids in a cataractous lens is also inadequately explained.

A slight advance in our knowledge of the nature of the thermostable residue of the lens is made by the proof that the protein  $\beta$ -krystallin acts alone as a T.S.R. and in proportion is quite as active as the T.S.R. prepared from the

whole lens. Since the other lens proteins are devoid of this power, it appears that  $\beta$ -krystallin is the active constituent of the lens T.S.R. It is significant that in this respect Jess observed a marked decrease in the amount of  $\beta$ -krystallin in a cataractous lens as compared with a normal lens. This was associated with a diminished nitro-prusside reaction. What actually happens to  $\beta$ -krystallin in cataract is unknown. It may escape from the lens, or be destroyed *in situ* by such agents as ultra-violet light

A minor effect of ultra-violet is the decrease in the power of the lens to oxidise certain organic acids. This deleterious effect on the enzymes of the lens may also occur *in vivo*

In conclusion it must be clearly stated that the experiments recorded in this paper are only a preliminary study of the problem. Much more experimental work is needed if the chemical changes in a cataractous lens are to be explained

#### SUMMARY

1. A fresh ox lens has a definite oxygen uptake, which is increased in the presence of glutathione, and markedly increased if both linseed oil and glutathione are present.

2. The power of the lens to utilise oxygen is considerably decreased by drying the lens, and it is absent after dialysis of the lens. Addition of a few milligrammes of glutathione to a suspension of dried or dialysed lens restores the oxidation power to normal.

3. A thermostable residue can be prepared from the lens, which has no oxygen uptake of its own. With glutathione, however, it gives a typical oxygen-uptake curve. It reacts with linseed oil and glutathione together, and develops a large and rapid oxygen uptake.

4. Only one of the three proteins in the lens can function as a thermostable residue, viz.,  $\beta$ -krystallin.

5. The average glutathione content of an ox lens, as estimated by Tunnicliffe's method, is 0.305 gm. per cent.

6. Exposure to ultra-violet light, or to heat rays, causes a decrease in the glutathione content of a fresh ox lens. The effect of ultra-violet is more marked than that of heat.

7. The lens is able to oxidise certain organic acids, but its power to do so is decreased by exposure to ultra-violet light.

In conclusion I wish to thank Sir William Hardy and Prof. Sir Gowland Hopkins for suggesting this work, and for their interest and advice. I am also

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## *The Hydrogen-Ion Concentration and the Oxidation-Reduction Potential of the Cell-Interior: a Micro-Injection Study.*

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# INTRODUCTION

It has been recognised since the middle of the eighteenth century that one of the most fundamental characteristics of living organisms is their capacity

to oxidise substances incapable of oxidation at ordinary temperatures, but no qualitative estimation of this power of oxidation was carried out until the time of Ehrlich, whose classical experiments on the injection of methylene blue into the intact animal revealed the fact that certain organs seemed to have a higher reducing power than others. Later, much histo-chemical work was done. Numerous observers studied the effect of staining tissues and cells in reagents which indicated by their colour whether they were reduced or oxidised.

Several such indicators were used by the earlier workers, especially  $\alpha$ -naphthol and pyronin and  $\alpha$ -naphthol and gentian violet, but the two chief methods were the intracellular formation of an indophenol, introduced by Rohmann and Spitzer in 1895 (20), and the oxidation of the leucobase of methylene blue, introduced by Unna in 1911 (23). In the latter case the cell was placed in a solution of the completely reduced dye, and the conclusion was that wherever the blue colour appeared, there the cell had been able to oxidise it. The indophenol method depended on the actual formation and precipitation of a dye in the cell by an oxidative condensation. The original reactants used were dimethylparaphenylenediamine and  $\alpha$ -naphthol, but various later workers modified this by using other phenols and other aromatic amines, so that indophenols of different colours were produced.

In 1909 Schültze (22) discovered that by a modification of the original technique the reaction might be applied to leucocytes and used as a differential diagnosis for the myelogenous leucæmias. From thenceforward the subject passed into the hands of more uncritical workers, with the result that a large and unsatisfying literature developed, mostly in clinical journals. Lillie, however (13), studied the indophenol reaction in the erythrocytes of the frog. He came to the conclusion that much the greatest formation of indophenol went on at the nuclear membrane, and he was able to increase this by passing a series of weak induction shocks through the cells during the process. It has never been certain whether this work really pointed to the nuclear membrane as the seat of intracellular oxidation, because staining effects could not be entirely excluded, although, as the dye is insoluble, it is less likely to be carried from place to place.

The rongalit-white method of Unna was still less satisfactory. It does not follow that the parts of the cell which stain blue in his treatment are really the places where oxidation has gone on, they may simply be examples of ordinary basic staining. Unna's contention that the blue parts of the cell (usually the nucleus) are oxidation-places still remains open to the objection

that the dye may have been oxidised anywhere and then stained basophile-places in the ordinary way

But the question of localised oxidation-places in the cell, upon which attention had mostly been concentrated in the earlier work, was really secondary in importance to the question of the actual absolute oxidising power of the cell. The fact that cells behaved differently towards the dyes used could mean no more than that the dyes were different, and since no zero of oxidation-potential existed, there was nothing with which to relate these differences. Drury in 1911 went into the matter (11), and showed by experiments of a purely physical nature that the condensation of a so-called test-substance for active oxygen, or a simple basic dye, not only will not take place on to a surface wholly devoid of oxygen, but is actually hindered by the existence thereon of a film of oxygen.

In a note to Drury's paper, W B Hardy made the following exceedingly important remarks: "It must be remembered always that an oxidation place is also a reduction place. It is to be called the one or the other according to the particular zero which is chosen. A convenient zero is the chemical potential of atmospheric oxygen, and a place would be an oxidation place if oxygen whose chemical potential is equal to or greater than atmospheric oxygen is condensed there to the intramolecular state. Such a region would then be a reduction place for chemical compounds in which the oxygen potential is equal to or greater than atmospheric oxygen, and an oxidation place for substances in which it is less than that of atmospheric oxygen. In the absence of some agreement as to the zero-point, the discussion is likely to be as confused in the future as it has been in the past." In other words, little meaning can be attached to experiments with indo-phenol, rongalit-white, Würster's tetra-substance, etc., for even if it could be shown that their effects were not due to simple basic staining, they yet cannot be related to any physical basis—in fact, to any zero.

The matter was left in that state for the following ten years, except that in 1919 Menten (15) showed that a positive result was obtainable with the Röhmann-Spitzer reagent *in vitro*, with emulsions of lecithin and cholesterol, under conditions which precluded the presence of an oxidase in the ordinary sense. But in 1923, Clark and his collaborators (3, 4, 5, 6, 7) published a most important series of papers. From a practical point of view their achievement has been to prepare and study dyes, which, at a constant hydrogen-ion concentration, change colour according to oxidation-reduction potential.

Employing the conception that reduction really consists in a transfer of

electrons from reductant to oxidant, Clark was able to measure, in terms of electrode potential, this electron-fugacity, or electron-escaping tendency. As a symbol for reduction-intensity, he chose the expression  $rH$ , to indicate an analogy with hydrogen-ion concentration,  $pH$ , an analogy which he found to be very marked as his work proceeded. Just as  $pH$  refers to intensity of acidity as opposed to the total amount of acid present, so  $rH$  refers to intensity of reduction as opposed to the total amount of reductant present. The only difference is that while  $pH$  means the negative logarithm of the hydrogen-ion concentration,  $rH$  means the negative logarithm of the hypothetical hydrogen pressure in equilibrium with the oxidation-reduction system in question. Both  $pH$  and  $rH$  are intensity and not capacity factors

By making a large number of electrometric observations on certain dyes, such as the sulphonated indigos and brominated indophenols, Clark and his collaborators have been able to prepare a series of oxidation-reduction indicators, of which it is possible to say that their curves, from complete reduction to complete oxidation in each case, are related to definite hydrogen pressures. They can accordingly be arranged in a table, and, as a matter of fact, a large part of the complete range between pure hydrogen and pure oxygen at atmospheric pressures has already been mapped out

Another analogy between acidity and oxidation-reduction potential is found in the fact that when the concentrations of the reactants are approximately equal, the addition of a given amount of either causes very little change in the system. This in the case of hydrogen-ion concentration has long been known as buffer action, and Clark proposes the term "poising action" for the phenomenon in which oxidant and reductant take the place of acid and salt.

It is to be noted that the  $rH$  is the negative logarithm of the hypothetical hydrogen pressure corresponding to a certain electrode potential at a known and constant hydrogen-ion concentration. The effect of  $pH$  on  $rH$  is most profound, and exists mainly because changes in  $pH$  affect the ionisation of the oxidant and reductant, and hence their active mass. Accordingly, when it is desired to know the  $rH$  of a given system, it is necessary first to know the  $pH$ .

It is obvious what an advance this represents on the work of Rohmann and Unna. We are now in possession of dyes, which, according to their colour, can be related to units of exact physical meaning. The problem was, therefore, to determine, first, the  $pH$  and then the  $rH$  of the cytoplasm of some cell. *Amæba proteus* was chosen.

The best method of attacking this problem seemed for two reasons to be

the use of the technique of micro-injection, much developed lately by Chambers (2). In the first place, with the organism in question, *Amœba proteus*, and probably with many other cells, this is the only way of introducing the dye into the uninjured cell, as the ectoplasm is impermeable to it. In the second place, upon injecting a small amount of the dye into the cell-interior, one can make an immediate observation, whereas staining methods are usually slow, and there is the possibility that with lapse of time and the presence of large excess of the dye, the initial equilibrium may be upset and an abnormal one established.

#### GENERAL METHODS AND TECHNIQUE

The micromanipulator apparatus used was very similar to that described by Chambers (2), but it had a few modifications. A description of it is to be found in Chambers' paper; it is sufficient to say here that it consists essentially of three rigid metal bars connected at their ends to form a very compressed Z-like figure by resilient metal acting as spring hinges. The pipette is carried in a holder on one bar, and by the action of three extremely fine screws the bars can be forced apart; in this way the tip of the microneedle or pipette is made to move very small distances in all three dimensions of space.

In Chambers' instrument one or two manipulators were clamped to the stage of the microscope or to a fixed pillar on the same basis. For injection Chambers used a Luer (hypodermic) syringe connected to a fine brass tube, which in turn was soldered to a glass shank placed in the pipette holder of the instrument. The pipette was drawn out of narrow tubing and cemented into the shank. By fixing the syringe into an old microscope, and using the milled heads on it to depress the plunger, delicate injections could be made. Our instrument was made for us by the Cambridge and Paul Scientific Instrument Company, and it has the following modifications:—

1. Both our micromanipulators were fastened to a pillar resting on the same heavy base as the microscope. At the bottom of the pillar there was a joint, which could be made fast by a set-screw, permitting all possible horizontal movements. This allowed the whole apparatus to be moved away from the microscope and swung round as desired. It also allowed of the freest possible movements of the stage, which was controlled by two diagonally set milled heads. The microscope was clamped down very firmly to the base.

2. It appears from Chambers' paper that his coarse adjustments in the horizontal directions were accomplished by sliding the needle or pipette in the holder, and in the vertical direction by sliding the post of the pipette carrier up



and down. In our instrument the pillar could slide backwards and forwards and round in any direction, while, in addition, we had a special screw for the coarse adjustment of up-and-down movements.

3. For the injection we used a hypodermic syringe connected by means of glass capillary tubing to a short rubber tube about seven inches long leading to the glass pipette. This rubber tube, which was sewn into a tightly fitting cambric coat, and was then closely bound with thin copper wire, was quite flexible and yet did not expand appreciably under the pressures used. By its aid movements of the pipette were greatly facilitated. Between the syringe and the pipette there were inserted two capillary three-way taps, one leading to a reservoir of boiled water, and the other opening to the exterior. By opening the former and raising the plunger the amount of liquid in the system could be increased, by opening the latter, it could be diminished. The syringe was fixed into an old microscope frame, after Chambers' manner.

4. In making the pipettes, glass tubing of about 8 mm. external diameter, to fit the holder, was drawn out into a capillary of from 1 to 2 mm. diameter. This capillary at a point about 8 cms. from the shank was then further drawn out in a microburner made from a hypodermic syringe as described by Chambers. In our apparatus, therefore, the shank and the pipette were all in one piece, and there was no cementing to be done. The shank was carefully wired into the rubber connection.

The making and turning up of the pipettes was carried out just as described by Chambers, except that we found that we could usually obtain pipettes of the desired type—tapering rapidly and with an opening of from 2 to 8  $\mu$ —directly by drawing out, without the necessity of breaking against a coverslip. A photograph of the whole apparatus is given in fig. 1.

The pipette was always filled just before the injection by connecting it to the syringe, introducing the tip into a hanging drop of the dye, bringing it into the field, and alternately applying and releasing gentle suction. Too strong and continued suction is a fatal error, as any particles suspended in the liquid become firmly lodged in the pipette end and block it irretrievably.

It was, of course, necessary, when injecting the pH indicators, to use "alkali-free" glass. We found that narrow combustion tubing worked well, though even with this the solution of a dye neutral when drawn in became, after a very short time, distinctly alkaline at the end of the column away from the tip. The same glass was also used for injecting the oxidation-reduction indicator.

The amœbæ were cultured in tap water containing a few boiled wheat grains.

They were washed once with distilled water before being placed singly in hanging-drops of distilled water. These coverslips when inverted formed the roof of a moist chamber. We found that injection was far easier if the amœbæ had attached themselves to the roof, and in order to bring this about the coverslips were always left protected from evaporation, lying drop upwards as long as was necessary for the complete settlement of the organism.

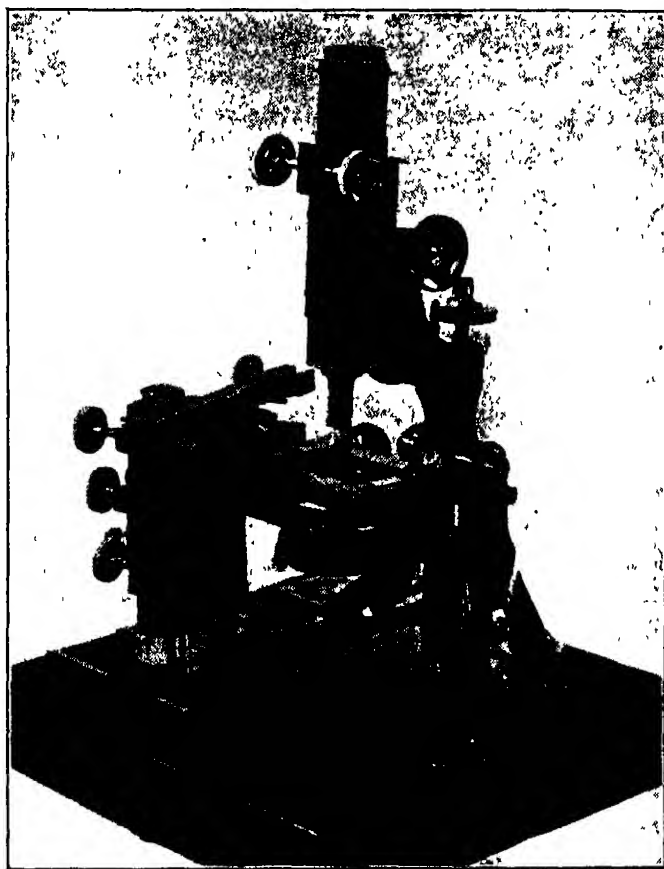


FIG. 1.

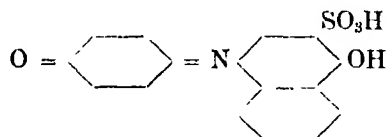
Nearly all our injections were performed under a Zeiss  $\frac{1}{4}$ -inch objective and a Watson eyepiece giving a magnification of 15. When using the Leitz double eyepiece its magnification was 5. The pipette was filled and the tip was quickly centred, using the coarse adjustments. A coverslip with hanging-drop was inverted over it, and the stage was moved until the amœba was immediately above the pipette tip. Then, using the fine adjustments, the

tip was raised until it just pierced the amœba. This moment could easily be ascertained, as a slight shock to the organism could always be observed when the tip got in, a sudden momentary cessation of streaming movements, which thereafter immediately began again. This shock was not visible if the tip merely pressed into the organism, causing an invagination, but without entering. When the pipette had entered the amœba, a few turns of the milled head over the syringe caused the injection, and the pipette was then at once withdrawn.

In order to make sure that our technique was satisfactory, and that we were not injuring the organism by the entry of the pipette, we made a few preliminary experiments with methylene blue. The results were good; the amœbæ appeared quite unharmed, and continued their streaming and pseudopodial movements. In some cases our observations confirmed Chambers, and there was seen coagulation of the protoplasm at the point of injection, but more often there was no sign of this, and the blue colour spread at once like a flash all over the cell.

The pH dyes chosen for injection were neutral red, phenol red, and brom-thymol blue. These appear to be the only easily obtainable indicators covering the physiological range which have a negligible protein error (*see* Homer (12) and Clark and Lubs (8)). In arranging the concentration of the dye solution to be injected, we chose the most dilute solution which, when an amount of it equal to about one-fifth of the total volume of the cell was injected, gave an observable colour. With neutral red a concentration of 0.2 per cent. was sufficient, with phenol red 0.5 per cent., and with brom-thymol blue about 0.8 per cent.

The oxidation-reduction indicator used was the sodium salt of 1-naphthol 2-sulphonic acid indophenol, a sample of which was made for us by British Drug Houses, Ltd. This dye is particularly suitable in several ways: first, from Clark's paper it seemed likely that its range would be in the neighbourhood of what was physiological; secondly, its formula shows it to be an acidic dye



so that there is little fear of the strong basic staining seen with methylene blue. Moreover, coagulation of the cytoplasm is not to be feared. Thirdly,

we found that it is a vital stain. *Paramœcia* placed in a very dilute solution (about 0.02 per cent.) were stained deep pink after a few minutes and continued actively moving for 20 minutes or more. As will later be described, injected amœbæ may survive as long as 20 minutes and show pseudopodial activity. Fourthly, we were able to show that the protein error of the dye is inappreciable. Two series of tubes were prepared, both containing the dye reduced to various stages by addition of increasing amounts of titanous chloride. In one series whipped egg albumen was added, so as to make the total concentration of protein up to 6 per cent.; in the other an equivalent amount of water was added. No difference in colour between corresponding tubes in the two series could be detected.

#### EXPERIMENTAL RESULTS.

##### *A.—Determination of the Hydrogen-Ion Concentration of the Cell-Interior.*

1. *Staining with Neutral Red.*—Neutral red is the only one of the pH dyes applicable which passes through the ectoplasmic membrane of *Amœba*. Several experiments were therefore carried out in which a considerable number of amœbæ were stained with neutral red *intra vitam* in various concentrations, removed into clean water and there carefully examined. Most of the animals were stained by immersion for 1½ hours in 5 c.c. of water containing six drops of 0.02 per cent. neutral red. The ectoplasm and endoplasm were stained always faintly yellow, and the numerous small granules in the latter were yellow or colourless.

To find the exact tint Pantin's method (17) of having a row of standard test-tubes filled with buffer solutions containing indicator interposed between the light-source and the microscope was employed. Such a row is seen in the photograph of the micro-injection apparatus in fig. 2. In this way the images of the tubes are thrown on to the microscope field and their tints can be compared with that in the interior of the amœba. The colour corresponded in all cases to a pH of 7.6. In none of these experiments could we detect any local formation of pink colour at the tips of the pseudopodia, as was described for certain marine amœbæ by Pantin (17). In one case one amœba put out an active pseudopodium along the surface of an inactive amœba. Even with this direct comparison no difference at all could be detected between the colour of the two. In a letter he tells us that only one species of marine amœbæ will show the change. In our amœbæ the nucleus was small, and did not show up clearly when stained with neutral red.

2. *Injection of Neutral Red.*—The results of the experiments in which neutral

red was injected by a micro-pipette of about  $4\ \mu$  diameter at its tip, agreed very well with the results of staining work. Exactly as is described by



FIG. 2.

Chambers (2) when neutral red is injected, the point of entry of the dye is marked by a clump of coagulated cytoplasm, and after about 30 seconds the endoplasm appears to contract, with the result that there is seen an effect of widened ectoplasm. It looks as if the ectoplasm had squeezed out much of its water, which then collected between it and the ectoplasmic external membrane. If the amount injected has not been more than a quarter of the total bulk of the amoeba, the endoplasm eventually expands and the amoeba is filled with small pieces of the coagulum first formed. But at the same time there is a marked yellow tint to be seen over the whole of the contracted endoplasm around the coagulated area, and this persists if the amoeba recovers.

On many occasions the neutral red in the pipette was pale pink, and, owing to the slight acidity of the hanging-drop, some of it which escaped there was pink also, but as soon as the dye entered the cell the dark red coagulated portion was seen surrounded by a faintly yellow zone. It was mentioned above that although methylene blue, another basic dye, sometimes formed a similar coagulum at the point of injection, it did not always do so, and spread like a flash all over the cell. Such a flash effect was never seen with neutral red.

3. *Injection of Brom-Thymol Blue.*—This indicator is distinctly more toxic for amœbæ than is neutral red. It is unusual for an amœba to last without cytolysing more than a couple of minutes after the injection of the dye. Nevertheless, this time is ample for the observation of a series of colour changes, and the results are quite confirmatory of what has been said already.

A description of a typical experiment will bring this out clearly. A micro-pipette of about  $2\ \mu$  diameter at the tip, filled with brom-thymol blue, was brought underneath a hanging-drop containing an amœba adhering to the under surface of the coverslip. The dye was green in the pipette, corresponding to a pH of 6.8 or less, and when a little of it was allowed to escape into the drop, it was seen to be green also. The pipette was then raised and brought under the amœba. On injection the dye rapidly permeated the whole endoplasm and ectoplasm, turning as it did so a definite pale blue, corresponding to a pH of 7.6. The penetration was not so rapid as that of the methylene-blue flash effect, but not much slower. After 70 seconds the amœba began to cytolysise, and as it did so the blue colour turned green and finally yellow.

This liberation of acid was an invariable characteristic of cytolysis, and was observed with all three dyes. The blue colour of brom-thymol blue in the amœba was always observed, though the dye injected was not always as acid as 6.8. Experiments showing such a striking change are difficult to bring about, for, even using combustion glass, the alkali diffusing out has a marked effect on dye in a tube  $3\ \mu$  in diameter. Accordingly, the amœba has to be injected just at the right moment. After the pipette is filled, the dye in it is steadily going more alkaline, and a suitable amœba may not appear until it is too late.

A few of the amœbæ injected with brom-thymol blue behaved in an atypical manner. One cytolysed until about half its granules had been lost, then suddenly healed up and remained stationary, having a blue colour of about pH 7.4. Two hyaline vesicles of ectoplasm outside it had a tint of 6.9 and 7.6 respectively. Another behaved in the way usually associated with neutral red, i.e. its ectoplasm swelled out and its endoplasm shrank, the latter having a blue colour of 7.6, and the former colourless. Cytolysis occurred after 20 seconds, whereupon there supervened the usual burst of acidity.

4. *Injection of Phenol Red.*—Phenol red, when injected into an amœba, produces effects somewhat like those resulting from the injection of brom-thymol blue. There is no coagulation at the point of injection and the dye penetrates rapidly all over the cell, taking up the colour corresponding to the pH of the interior. After a period which is very variable, the amœba cytolyses,

becoming very acid as it does so. Pseudopodial movements while the dye is in the cell are rare.

In a typical experiment, the indicator was yellow in the pipette, implying a pH of 6.9 or less, but as soon as it entered the amoeba, it turned a definite pink, corresponding to pH 7.6. In many cases a little dye was allowed to escape from the micro-pipette as it was withdrawn from the hanging drop, and the amoeba then stood out coloured pink against a background of yellow. This, like the analogous phenomenon in the case of brom-thymol blue, was observed again and again. It must also be remembered that the Leitz double eyepiece allowed of the simultaneous observations of both collaborators.

The great increase of acidity on cytolysis was equally evident in the case of this dye. Just before the amoeba was going to cytolysise, the colour would fade to a pale orange, and this would turn deep yellow as cytolysis took place. All these colours, both in the cases of this dye and of brom-thymol blue, were ascertained by the use of Pantin's suspended test-tube method.

Here, again, a few amoebæ did not behave like the rest. In one case, healing of the ectoplasm occurred after a slight amount of cytolysis had taken place, after which the pseudopodial movements of the cell were very active, and the remaining part was again injected. Only after two and a half minutes did it cytolysise completely; and then it exploded, the colour immediately changing from pink to yellow.

When phenol red was used, two differences from brom-thymol blue were noticeable. In the first place the amoebæ seemed to be able to exist longer with the dye in them, some as much as five or six minutes before cytolysing. Secondly, when they did cytolysise, it was often with explosive violence, the ectoplasm lashing about in all directions, the granules streaming out centrifugally, and the colour changing in a flash from pink to yellow.

#### 5. Discussion.—

#### *Number of Experiments done on pH.*

		Brom-Thymol Blue	Neutral Red		Phenol Red.	Total.
Successful Unsuccessful (immediate cytolysis, or other reasons)		Injections. 14	Injections 27	Staining 11	Injections 29	81
		6	3	0	0	9
		20	33	11	29	90

It is seen from the above table that out of 90 experiments, 81 were confirmatory of each other, or a little under 90 per cent. Each single phenomenon was confirmed many times. There would seem to be no doubt, then, that the *pH* of the interior of the amœba studied by us is in the very close neighbourhood of 7·6. It must be understood that this figure is only of immediate application, and refers to fresh-water *Amœba proteus*, growing in wheat cultures and under definite conditions of temperature and light. For other conditions the figure might not apply.

In our experiments we found no trace of a localisation. The nucleus was so obscured by the granules that we can say little about it, but so far as we could see, the cell appeared to react as a whole. How does this value of 7·6 compare with previously ascertained values for intracellular hydrogen-ion concentration? Crozier (9) in 1923 studied the Nudibranch, *Chromodoris zebra*, which contains a natural indicator, and found that it must be less acid than 5·6, while with other pigmented animals he found them to be more acid than sea-water (8·2), *i.e.* at about 7·4. This is not far from our value.

Ritchie (19) investigated the change in *pH* caused by mammalian muscular contraction. Pachstein had found in 1914 (16) that the juice of fatigued muscle was *pH* 6·9, but Ritchie, who used a careful technique, found that there was no difference before, during, and after activity, and that the *pH* always varied between 7·5 and 7·8. It is interesting in view of our failure to find any local concentration of acid in pseudopodial activity.

Atkins (1) stained *Pleurobrachia pileus*, *Clytia johnstoni*, and *Tiara pileata* in neutral red, and found that they all showed a *pH* of 7·0. *Clytia* and *Tiara* he found to be permeable to brom-thymol blue, and the former gave a value of *pH* 6·6, while the latter was 6·4 in the motile parts and 7·2 for the non-motile umbrella. Vorticella, he found, showed a tint with neutral red corresponding to *pH* 7·0. Pantin (17), studying several types of marine amœbæ, found that the endoplasm gave tints with neutral red corresponding to *pH* 7·6 to 7·8, the ectoplasm 7·1 to 7·3, and in some species the active pseudopodia to *pH* 6·8. In the resting condition the difference between endoplasm and ectoplasm was less marked.

Vlès (24) gives a list of other experimental findings as regards the *pH* of the cell interior of plants, etc., and his paper should be consulted for the literature. He himself, working on eggs of *Paracentrotus lividus*, found that they had for the most part an internal *pH* of from 5·6 to 6·0, while on cytolysis the *pH* was raised to 7·0 or 8·0. His method was to crush the cells in drops of the dyes.

Reiss (18), a collaborator of Vlès, obtained values of about *pH* 5·0 by the



same method for Echinocardium, Styelopsis, Aplysia, Nereis, Sabellaria and Trochocochlea eggs, and for protozoa such as Cryptochillum and Colpidium. He reported that the nucleus seemed to follow variations in the acidity of the external medium much more closely than did the cytoplasm.

Lastly, Schmidtman (21), using Péterfi's micromanipulator apparatus, injected small grains of neutral red, brom-thymol blue, and *p*-nitrophenol into muscle and liver cells. By comparison with drops of buffer solutions treated in a similar way, this method gave a result of pH 6.7.

### *B. Determination of the Oxidation-Reduction Potential of the Cell-Interior.*

1. *Injection of the Completely Oxidised Dye.*—Before the completely oxidised dye could be injected into the amoebæ, two problems of technique had to be faced. In the first place, although the setting up of a series of pH indicators is a comparatively easy matter, and consists simply in adding a constant amount of indicator to a series of buffer solutions, the setting up of a series of *r*H indicators is not so simple. The dye has to be brought to the desired degree of reduction by titration against a standard reducing agent in an atmosphere of nitrogen. Secondly, when a change of tint is to be observed, the thickness of the comparator tube does not much matter; but in the case of an oxidation-reduction indicator which changes from red or blue to a colourless leuco state, the change to be observed is only in depth of colour, and then the thickness of the layer looked through makes all the difference.

These difficulties were solved in the following way (see fig. 3):—The titration vessel, A, was a boiling-tube. Into it there dipped a tube  $\alpha$ , which was connected to a nitrogen cylinder, and an outlet tube  $\beta$ . The two burette tips,  $\gamma$  and  $\delta$ , were connected respectively to the burettes G and D, the former of which contained oxygen-free distilled water, and the latter the reducing solution of titanium chloride. Distilled water was supplied as required from the bottle E, and both burettes were connected through the tubes F and H to an aspirator containing nitrogen. The tube D was filled from the reservoir J. Into J there also dipped the tube of a straight-sided separating funnel K, and the two pipes  $\eta$  and  $\epsilon$ . By suitable manipulation of the taps X and Y, the stream of nitrogen from the cylinder could either be made to pass successively through the buffer solution in J, the titanium solution in K and the distilled water in E, or it could be used to force the liquid in J up into the burette D.

The usual procedure, adapted from Clark (5), was as follows:—1.75 grams of titanium chloride prepared with proper precautions from the commercial solution were dissolved in 30 c.c. of air-free M/5 citric acid, and 20 c.c. of this

placed in K. Into J were placed 100 c.c. of N/5 di-sodium hydrogen phosphate, 64.6 c.c. of N/5 sodium hydroxide, and 15.4 c.c. of distilled water. When

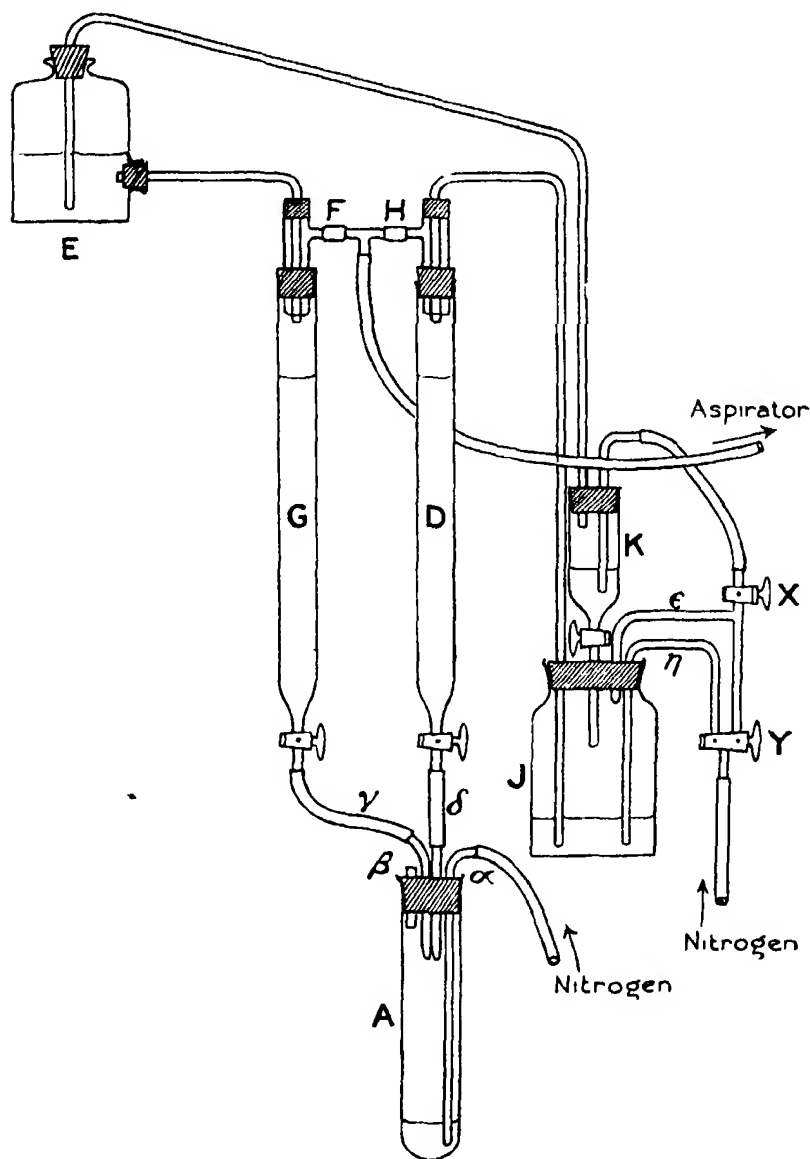


FIG. 3.

nitrogen had been passed through the whole system for 20 minutes or more, the titanium was allowed to run down into the buffer solution and mix with it.

The advantage of this technique is that the titanium buffer solution, which does not keep well, can be used in the titration as soon as it is made up. We thus had a solution of titanous chloride dissolved in buffer solution at 7.6 (the reaction we had previously found for the cell-interior) and containing compensation alkali to neutralise the acidity of the titanium chloride. All that needed to be done was to titrate it against the dye

For this purpose 5 c.c. of 1 per cent. sodium 1-naphthol, 2-sulphonate indophenol made up in phosphate buffer solution at pH 7.6 was placed in the titration vessel and nitrogen run through it for some time. When everything was ready, the titanium solution was run in until the stage of complete reduction was reached. Trouble was encountered here with regard to the end-point. It was essential to use the dye of this strength, for it had to be comparable to the dye injected, and preliminary injection experiments had shown what strength combined least toxicity and greatest visibility. Clark (5) had used very much weaker dye and titanium, and had found the end-point to be an almost invisible yellow tint. Eventually we found that the end-point could be well seen by transmitted light. We carried out the titration in front of an electric lamp, of daylight glass, and observed that a definite transition from claret colour to faint dirty brown marked the attainment of complete reduction.

Having then ascertained how many cubic centimetres of titanium solution were required to bring the dye to complete reduction, it was only necessary to add various fractions of this figure to other samples of dye in order to get standards of any desired percentage reduction. In each case, when the titration was completed, an amount of distilled water was added from the burette G sufficient to compensate for the dilution which would have occurred if the sample had been taken to complete reduction.

In the meantime a supply of capillary tubes of combustion glass, measuring approximately 50  $\mu$  internal diameter—not far from the diameter of the amoeba—had been prepared, and one of these was passed down the tube  $\alpha$  into the prepared dye. At once the indicator ran up it, pushing in front of it an area of redness, due to contact with the external air, but being for the most part quite unaltered. After a minute or so the tube was removed and broken off into small pieces about  $1\frac{1}{2}$  inches long, the ends of which were quickly sealed with collodion. We thus prepared a series of “micro-test-tubes,” which corresponded to varying degrees of reduction, and which could be placed on top of the coverslip beside the injected amoeba.

The following description of a typical injection experiment illustrates best

what the appearances were:—The amoeba having settled on the coverslip, the micro-pipette was brought underneath it and an amount of the dye (1 per cent.) equal to rather less than a quarter of the total bulk of the amoeba was injected. The dye penetrated the whole of the protoplasm, leaving at first a small zone of uncoloured material, rather wider than the ectoplasm, but not sharply defined, all round the outer part of the cell. This uncoloured zone only persisted for a few seconds, and thereafter the entire cell was coloured. No liquefaction of the protoplasm appeared to take place and pseudopodial movements were seen on several occasions, though more often the cell retracted such pseudopodia as it had out and settled down into a more or less spherical shape. There were never any attempts at extrusion of a blister of coloured cytoplasm, and the contractile effect seen with neutral red was never observed with this dye. Cytolysis might take place at any time, but often the cells remained apparently uninjured for 10 or 20 minutes. When cytolysis occurred, it was always a rather slow process, and no explosions were ever seen.

But the colour change was the most interesting. Immediately after injection the cell was a deep red; within the first 60 seconds this colour rapidly faded to a very pale but quite distinct pink. That this change was not caused by diffusion into the drop was clear from the fact that no trace of colour appeared outside the periphery of the cell. After it had attained to this faint pink, the fading went no farther for some time. In no case did the dye in our experiments ever go quite colourless in the cell, except immediately before cytolysis. Frequently, for three or four minutes or longer, the faint pink would be maintained, with no alteration, and appeared to be unaffected by streaming movements when these took place, later we sometimes observed further very slow fading.

By the use of the micro-test-tubes placed on top of the coverslip, we found that the position of equilibrium at the standard pink corresponded to a reduction of from 15 to 30 per cent. For reasons which will be given later, the exact determination of the point of reduction was a little difficult, but the cardinal fact was that the equilibrium position lay within the range of the indicator.

Cytolysis, when it did take place, was accompanied by a most interesting phenomenon. The indicator, which previously had been of the faint pink colour, suddenly became quite colourless as the amoeba cytolysed. That this was not due to the dissipation of the dye into the drop was very clear, for although the amoeba had suddenly gone colourless, there was not the slightest

trace of any corresponding reddening in the near vicinity in the drop. The only explanation seemed to be that, when cytolysis took place, not only was there a burst of acidity, but also a burst of reducing activity. Substances which needed oxidation must have been liberated and the dye reduced to satisfy them. An exactly similar phenomenon has been described by Pantin (17) in his paper on marine amœbæ. "During the process of cytolysis," he says, "the colour of the neutral red faded entirely away, leaving the spheres of protoplasm colourless." "This fading or bleaching of the neutral red was quite different from the change of the indicator from red to yellow in alkalis; the observations suggested that the stain was chemically altered, possibly by reduction or oxidation." It is unnecessary to point out how well these remarks fit in with our results.

The increase in acidity upon cytolysis might, of course, be itself the cause of the increase in reducing activity seen then, if enough acid was liberated. If the  $pH$  did not decrease too much, however, the reduction-intensity increase could not be accounted for in this way. In order to test this point brom-cresol purple was used as an indicator. This dye is purple from  $pH$  6.8 upwards, and yellow from 5.2 downwards, so that cytolysis might produce a yellow or yellowish colour. 0.6 per cent. brom-cresol purple was accordingly injected into a number of amœbæ. In no case was there the slightest sign of a yellow tint, so we conclude that on cytolysis the acidity does not go below  $pH$  6.0.

Now from Clark's tables (5) it is to be seen that for 1-naphthol 2-sulphonic acid indophenol at  $pH$  6.98, 20 c.c. of the titanium solution produced a percentage reduction of 60.98, whereas at  $pH$  8.9 19.5 c.c. of Ti produced a percentage reduction of 66.1. Consequently a  $pH$  shift of 2.0 only produces a change of 5 per cent. in the percentage reduction. The increase of acidity on cytolysis, which does not amount to more than 1.6  $pH$ , cannot then account for the increase in reduction intensity.

In all 42 completely successful experiments were performed with the oxidised dye.

Mention has already been made of the experiments in which methylene blue was injected into the cell. It was noticed that after about three minutes this dye was completely reduced, so that the organism was colourless. So far, only approximate data have been published by Clark (3) on the electrode potentials of methylene blue, and these show that it lies very close to 1-naphthol 2-sulphonic acid indophenol, though on which side is not yet certain. Our micro-injection results would lead us to expect that it will be

found to have a slightly more positive electrode potential than 1-naphthol 2-sulphonic acid indophenol, *i.e.* a higher *rH*.

2. *Injection of the Completely Reduced Dye.*—If the oxidation reduction system of the cell is, as a whole, of the reversible kind considered by Mansfield

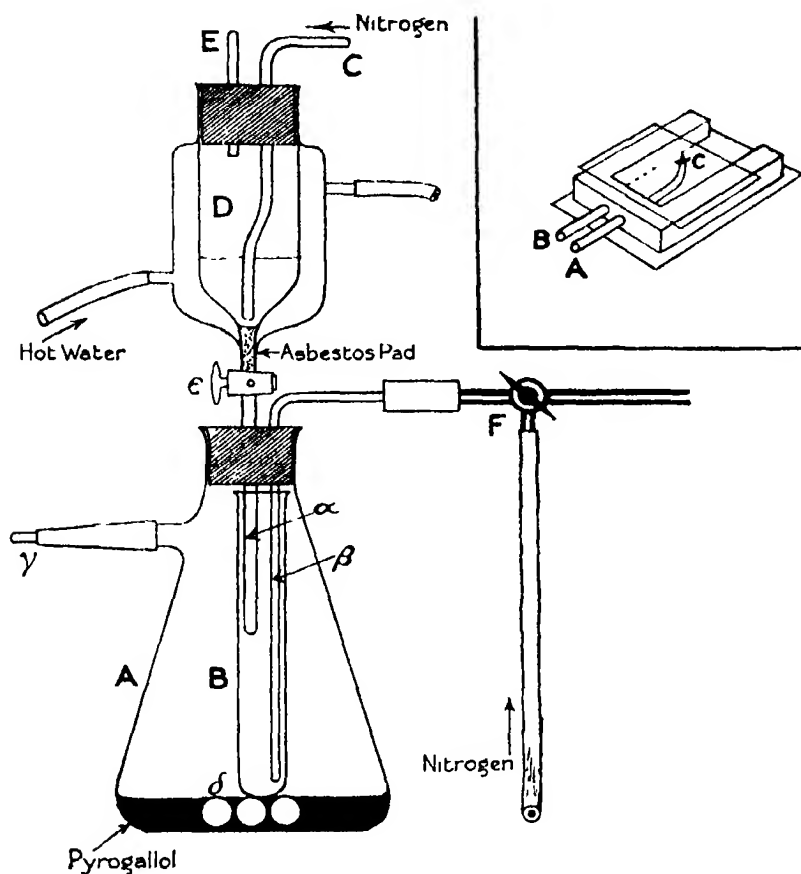


FIG. 4

Clark, then the completely reduced dye when injected should be oxidised to about the same point which it reached when reduced from the oxidised form.

The technical difficulties were here very great. The filling of the micro-pipettes with the reduced dye at first sight seemed impossible, but it was eventually accomplished in the following manner, with the apparatus shown in fig. 4:—A moderate-sized Büchner flask, A, was set up with a small quantity of pyrogallol at the bottom, to absorb traces of oxygen, and three or four pieces

of thick glass rod,  $\delta$ , to act as a stand for the test-tube B. Through the cork there came two tubes, a narrow one,  $\beta$ , connecting to a three-way tap, F, and farther on to the moist-chamber of the microscope, and a wider one,  $\alpha$ , which was part of the apparatus D. This consisted of a straight-sided separating funnel, furnished with a jacket through which hot water or steam could be passed. Into the top of D there entered through the cork the tube C, carrying in nitrogen, and an escape-tube was placed at E.

The inset shows the disposition of the moist-chamber. B is a tube carrying in a quick stream of nitrogen, so that the chamber contains little air, and A is the narrow tube coming from the apparatus described above. A is turned up at the end so that a drop of reduced dye is deposited on the under-surface of a coverslip. Around its end it has a ring of vaseline (c) to prevent the drop from flowing back on the outside of the tube on to the floor of the moist-chamber.

The course of an experiment ran as follows — A small piece of porous pot was placed in D, just above the tap  $\epsilon$ , and a column of well-washed asbestos was filtered on to it from above, so as to block up the tube as far as the broad part. Boiled water was now filtered through the asbestos pad into another Buchner flask, so as to remove the oxygen-containing water. Finally, nitrogen was passed through it. Ten c.c. of the dye solution (1 per cent.) was then run into D and a quantity of metallic zinc powder added. The cork was placed in position and pure nitrogen passed through it, while it was warmed with a stream of hot water in the outer jacket.

We tried platinised asbestos, but abandoned it because it formed a 'cake' round the base of the apparatus, and no amount of shaking would keep it properly dispersed. A more active catalyst we found to be diatomised brick with platinum deposited upon it, but this also we abandoned, for we found that the reduction carried out with its aid was irreversible. It apparently not only reduced the dye to its leuco-compound, but reduced it further and perhaps broke up the molecule. Zinc powder, on the other hand, at a temperature of about 85° C., reduced the dye to its leuco-base in about three minutes, although the dye was as strong as 1 per cent., and the reduction was obviously reversible, for if the least trace of air was suffered to get into the apparatus, the exposed parts of the solution at once went pink.

Meanwhile the Büchner flask had been filled ten times with pure nitrogen through  $\beta$ , and as many times evacuated by attaching a filter pump to  $\gamma$ . The tap F was now turned so as to close the system, and the tap  $\epsilon$  turned on so that the reduced dye filtered into the test-tube B. Taps  $\epsilon$  and  $\gamma$  were then

closed and  $\gamma$  attached to a nitrogen cylinder; pure nitrogen was then admitted through  $\gamma$  until there was a slight positive pressure. At this stage the amœbæ and micropipette were prepared, and when the latter was ready in the micro-manipulator to receive the dye, tap F was opened, whereupon the dye passed up  $\beta$  and along to the moist-chamber, where a neat drop was deposited on the roof. This could then be introduced into the micropipette in the usual manner.

Owing to the difficulties of the technique, the number of experiments done in this way were not nearly so great as with the simpler process of injecting the completely oxidised dye, but yet a sufficient number were performed to allow of the observation that although the cell sometimes became pink on injection, it did not always do so. We found that this was due to the fact that though in any two cases the dye in the pipette might be absolutely colourless, yet when the cell did not go pink the dye had been more completely reduced than in the other. The fact that cells would become a definite pink colour on injection of colourless dye was quite certain (more especially as the dilution which the dye would undergo in entering the cell was a strong adverse factor), but it seemed as though this would only happen when the dye was perhaps 90 per cent, instead of 100 per cent reduced.

We have not so far done a sufficient number of experiments to determine whether the colour produced in the cell in this way is exactly the same as that attained when the oxidised dye is injected, but observation leads us to think that it is not very far off. The explanation we would suggest as to why the cell can oxidise dye which is very slightly oxidised, and yet apparently cannot oxidise dye which is 100 per cent reduced, is as follows:—The cell, though probably well poised is not perfectly so, and it is to be expected that the injection of the reduced dye will lower its  $rH$  slightly, just as the injection of oxidised dye will raise its  $rH$  a little. In these circumstances, since the total range of the indicator is only 4  $rH$ , and the visible range under these conditions little over 2  $rH$ , only a very little would be needed to send the appearances right off the scale. The complete proof of the virtual reversibility of the cell system will only be attained when we inject into amœba another reduced indicator, of lower  $rH$ , such as one of the brominated indigos, in which case it would be expected to be completely oxidised. Or reduced 1-naphthol 2-sulphonic acid indophenol could be injected into an organism of higher  $rH$ , in which case it also would be completely oxidised. We hope to continue this work.

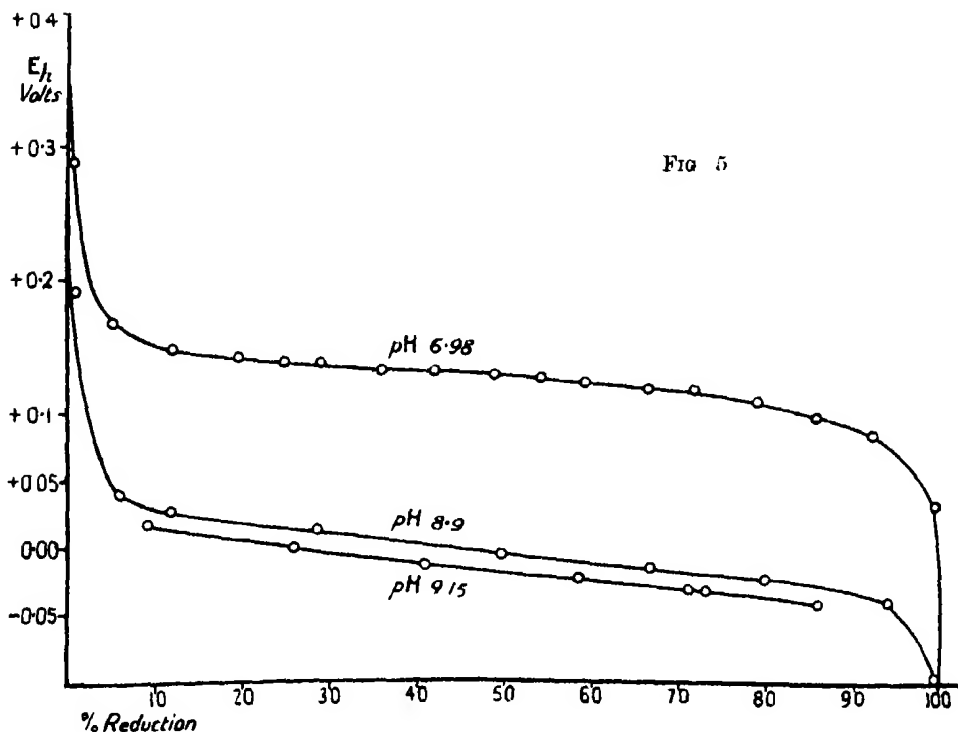
It might be objected that the oxidation of the reduced dye by the cell was



due to the presence of dissolved oxygen in the protoplasm. This objection is met, however, by the answer that what it is desired to measure, in all these experiments, is the resultant of all the factors there are in the cell which influence its oxidation-reduction potential, and if oxygen is present it must be considered as one of them.

3. *Calculation of the  $rH$ .*—As a general result of these experiments we concluded that the position of equilibrium which the oxidation-reduction indicator took up in the cell-interior was between 15 and 30 per cent. reduced. A nearer approximation is impossible for the following reason:—The shape of the titration curve of the dye, from complete oxidation on the one hand to complete reduction on the other, is S-shaped. The phenomenon of poisoning action is due to this fact, and the consequence is that when the dye is reduced to the neighbourhood of 50 per cent. its colour varies very little compared to the variation at the ends of the curve. It is much easier to tell whether the dye is 10 or 15 per cent. reduced than it is to tell whether it is 40 or 60 per cent. reduced.

Now in Clark's paper (5) the titration curves of 1-naphthol 2-sulphonic acid indophenol are given for  $pH$  6.98, 8.9, and 9.15, so that directly from his



figures there can be drawn a graph relating percentage reduction to electrode potential. If the axes are now altered so that the  $pH$  is plotted against the electrode potential at a constant percentage reduction, the electrode potential can be read off if the other factors are known. We naturally choose the percentage reduction of 30, for that is the figure derived from our experimental data. Fig. 5 shows the titration curves for different  $pH$ 's and fig. 6 the  $pH$

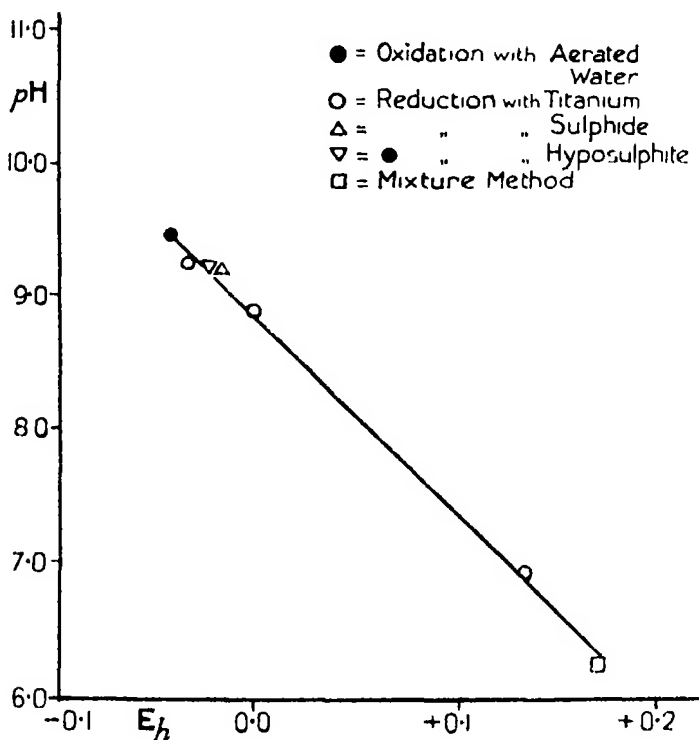


FIG. 6.

related to the electrode potential. Both these curves are constructed from data given by Clark (5).

Reading off, therefore, on fig. 6 from  $pH$  7.6, we arrive at an electrode potential value of  $E_h + 0.097$ . It will be remembered that  $rH$  is defined as the negative logarithm of the hypothetical hydrogen pressure, which would theoretically be in equilibrium with the oxidation-reduction system under consideration—hypothetical, because the units with which we are dealing are far smaller than any measurable hydrogen pressure. Accordingly it is possible to make a diagram where  $pH$  is related to electrode potential, and to place upon it a series of lines, each one corresponding to one  $rH$  unit. This Clark has done (5)

and his figure is reproduced here as fig. 7. Describing it, he says, "The zero point on the arbitrary scale adopted is the difference of potential between

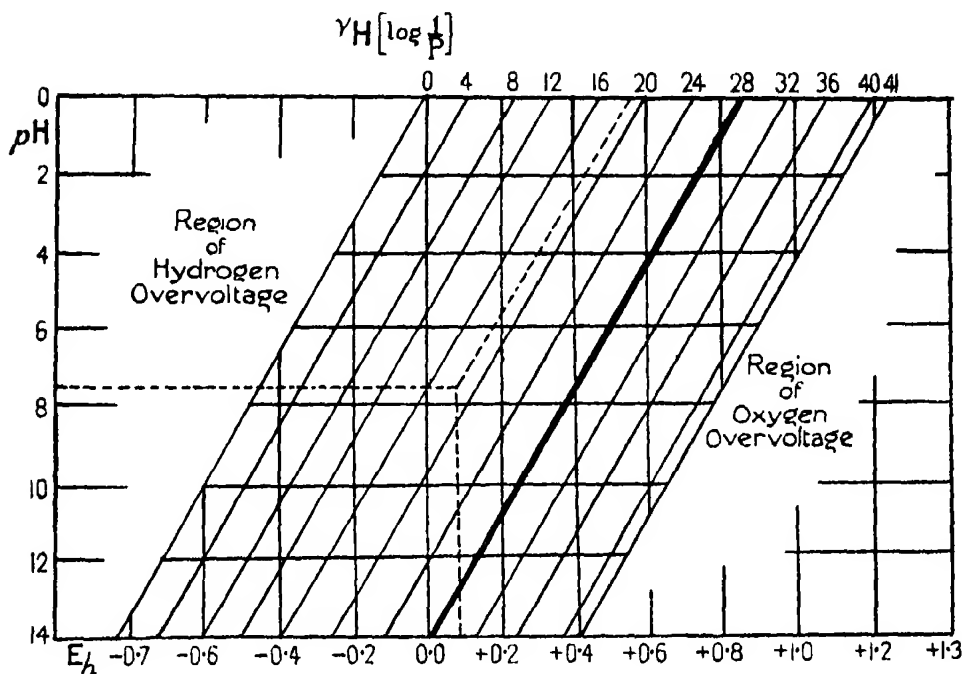


FIG. 7

electrode and solution in a normal hydrogen half-cell. The electrode potential equation for a hydrogen electrode is

$$E_h = -\frac{RT}{2F} \ln p - \frac{RT}{F} pH.$$

If the hydrogen pressure  $P$  is unity (in atmospheres),  $E_h$  will vary as  $-\frac{RT}{F} pH$ .

Then if the  $T^\circ$  is  $30^\circ C$ ,  $E_h = -0.06 pH$ . For each unit increase in  $pH$ ,  $E_h$  becomes more negative by 0.06 of a volt. This is shown in fig. 7 by the line starting at  $E_h$  0 and  $pH$  0. Parallel to this line may be drawn others showing the relation of  $pH$  to  $E_h$  when the hydrogen electrode is under a definite hydrogen pressure less than unity. It has been calculated that an electrode under one atmosphere pressure of oxygen should be 1.23 volts more positive than a hydrogen electrode under one atmosphere pressure of hydrogen in a solution of the same  $pH$ . This estimate fixes the position of the line on the extreme right of fig. 7."

It will be seen that oxidation-reduction neutrality exists at  $rH$  20.5. Apply-

ing now the value  $Eh = +0.097$  to this diagram, we find that it meets the  $pH\ 7.6$  line at a point between the  $rH$  values of 17 and 19. We therefore conclude that this represents the oxidation-reduction potential of the cell-interior.

#### GENERAL DISCUSSION

In arriving at this conclusion for the value of the  $rH$  of the cell, it has been necessary to make three important assumptions, and we must now consider their validity.

In the first place, we have supposed that the cell as a whole constitutes a reversible oxidation-reduction system of the type considered by Clark. Now it should be remembered that glutathione must play an important part in the oxidation-reduction equilibrium of the cell and, as was shown by Dixon and Quastel (10), the oxidation-reduction system reduced glutathione—oxidised glutathione is an anomalous one. The  $Eh$  seems to depend only on the concentration of the reduced form, the addition of the oxidised form having no effect, and this would seem to indicate that *in vitro* the oxidised glutathione cannot be reduced again. But probably this objection does not apply to the system *in vivo*, for in the tissues, as is well known, the glutathione can receive its hydrogen from donators and become itself reduced, the fact of the oxidation of the reduced dye by the cell is evidence in favour of our first assumption.

Secondly, we must suppose that the cell constitutes a poised system, for if this were not the case, the introduction of the dye would in all probability entirely upset the  $rH$ . As we have seen, Mansfield Clark used the term "poised" to denote a system in which the  $rH$  was kept fairly constant in spite of the addition of reactants, because the oxidant and reductant were present in proportion of about 50 per cent. of each. The use of the term may be extended to any system where factors, whatever they may be, are operating to keep the oxidation-reduction potential constant. Direct evidence as to the state of affairs in the cell is wanting, this is not surprising, as the conceptions are so new, but the interesting paper (which appeared when our own work was nearing completion) by Voegtlin, Johnson and Dyer (25) has perhaps some bearing on this point. These workers observed the time necessary for complete reduction of various oxidation-reduction indicators under standard anaerobic conditions by various tissues. They found that the time required for any given tissue depended on the concentration and the reduction potential of the dye—if these factors are constant, then the reduction time is constant for the tissue. They point out "that protoplasm is an extremely well-adjusted

system, and hence it is quite natural that the concentration of the substance or substances responsible for the reduction of the dyes by the tissues is quite constant under physiological conditions, and this may be the explanation of the remarkable agreement." Such a constancy in concentration of reductants as is indicated here would surely be a factor tending towards a poised condition of the cell

[Added April, 1925.—In connection with this point we would mention the classical work of Voit (26), who in 1866 came to the conclusion that the intensity of metabolism is not regulated by the oxygen supply. As Lusk puts it (14), the respiration does not cause or regulate the metabolism; rather, the reverse is the case. There must be in the cell some internal regulatory factor at work, so that the master-condition is demand and not supply. The significance of this is lessened by the fact that Voit worked on rabbits, whose blood is already nearly saturated with oxygen as it leaves the lung, so that increase of external hydrogen pressure cannot directly affect the cells of the tissues. Warburg, however, working on the developing eggs of *Echinus*, found that within wide limits of environmental oxygen concentration their metabolism remained the same (27). "Nun habe ich nachgewiesen," he says, "dass die Oxydationsgeschwindigkeit im Ei unabhängig vom Sauerstoffdruck, *d. h.* unabhängig von der Sauerstoffconcentration im Ei ist, während diese Geschwindigkeit durch Veränderungen anderer Bedingungen sehr erheblich beeinflusst werden kann." Here, there can be no question of the blood as intermediary

Another similar test case is that of Insects. Batelli and Stern (1a) found that for the cockchafer, *Melolontha vulgaris*, and for the common fly, *Musca vomitoria*, the intensity of the gas exchange was not much greater in 95 per cent. oxygen than in air. Exactly the same result was obtained by von Buddenbrock and von Rohr (1b) on *Dixippus morosus*, the stick-locust. In all these forms the tracheal system conveys the air directly to tissues, so that the complication of the blood again does not arise. We suggest, then, that the relative independence of the organism, as regards environmental oxygen pressure, may perhaps receive its explanation in terms of the poisoning action of an oxidation reduction system.]

The third assumption concerns the point in the reduction of the dye by the amoeba which we chose for taking our observations. We assumed that the rapid fading seen in the first 60 seconds was caused by the reduction of the dye to a point in equilibrium with the oxidation-reduction system of the cell. We consider that the very slow fading sometimes seen after the standard

pink is reached may be due to a gradual decrease in  $rH$  caused by the toxicity of the dye. Most of our observations, therefore, were taken from the end of the first minute till the fourth or fifth, during which time no perceptible fading was going on. This would seem to be as near as one can attain to the actual oxidation-reduction equilibrium of the uninjured cell.

We should like to discuss here one or two other observations recorded by Voegtlin and his collaborators. One of the dyes they used was 1-naphthol 2-sulphonic acid indophenol, which they found completely reduced by tissues *in vitro*. On the other hand, after injection into the living animal, the dye was found in the oxidised state in almost all the tissues examined. As we have seen, this dye is not completely reduced by *Amæba proteus*, except upon cytolysis; it is, of course, highly probable that the amœba has a very different reducing power from that of the tissues of the rat, but it is perhaps just possible that some of the reducing activity observed *in vitro* was the result of injury to the cut cells, of which, in slices less than 2 mm thick, there must have been a large number. In the same way, this might be a possible explanation for the difference in reducing activity found between young cancer tissue and the necrotic portion from the centre of the neoplasm. For the necrotic cells would have passed through the stage of increased reducing activity, and the reducing substances set free in them would have been already oxidised before the experiment began. These suggestions are quite tentative.

The results reported in this paper are somewhat isolated, since they all refer to a single type of cell. They cannot be considered in their full significance until they can be compared to other biological data. It is to be hoped that these will soon be available; indeed, a wide field is opened up for investigations along these lines. Such problems as the oxidation-reduction potential of egg-cells before and after fertilisation, of rhythmically contracting organisms, and of cancer tissue, at once suggest themselves. But in spite of this temporary isolation, we believe that the results given in this paper are of some interest, for they are, as far as we know, the first attempt to relate oxidation-reduction potential—a fundamental property of the cell—to a definite physical standard.

#### SUMMARY.

Subject to the assumptions discussed in this paper, the cell-interior of *Amæba proteus* has been found to have a hydrogen-ion concentration of approximately  $pH$  7.6, and an oxidation-reduction potential of between  $rH$  17 and 19.

We wish to thank Prof. Sir Frederick Hopkins for his continued encouragement and inspiration. One of us (D. M. N.) is indebted to the Food Investigation Board for a grant held during the course of this research.

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*Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen. Part X.—The Oxygen Intake during Exercise while Breathing Mixtures Rich in Oxygen.*

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In the present series (Part VII) A. V. Hill, C. N. H. Long and H. Lupton\* showed an interesting result with normal subjects, on the oxygen intake during exercise while breathing rich oxygen mixtures. They found that there is a considerable rise, reaching 50 per cent., in the maximal oxygen intake when breathing oxygen mixtures, as compared with breathing air; and, further, they calculated under such conditions the work done by the heart. This phenomenon has been re-observed, and some possible errors that might occur in the method are here discussed. The method used was that of the Douglas bag, and the expired air was analysed by the Haldane apparatus. The exercise was "standing-running."

The most serious error that might complicate or affect the results is that due to contamination, by air, of the expired gases. The following simple calculation is sufficient to show the large effect of such contamination by air if it occurred. Suppose that 118 litres of a 50 per cent. oxygen mixture be mixed with 2 litres of air; then calculate how much oxygen has apparently disappeared. The oxygen in 120 litres of the new mixture is  $118 \times 0.5 + 2 \times 0.2 = 59.4$  litres, so that the oxygen percentage is now 49.5 per cent. Thus, assuming equality of the inspired and expired nitrogen, apparently about 1 per cent. of oxygen has disappeared, *i.e.* about 1.2 litres of oxygen corresponding to the 120 litres of expired gas. Now assume that a subject records a ventilation of 120 litres per minute and a maximal oxygen intake of 3.8 litres breathing atmospheric air; and that no rise in the oxygen intake actually occurs, when breathing a rich oxygen mixture, but that 120 litres of apparently expired gas now consist of 118 litres of actual expired gas and 2 litres of air leaked in. The result obtained in such an experiment would give an apparent oxygen intake of 5.0 litres per minute. The following experiments, therefore, were performed with every possible care taken to avoid leakage. The mouthpieces were tested and found to be air-tight, while

\* A. V. Hill, C. N. H. Long, and H. Lupton, 'Roy. Soc. Proc.,' B, vol. 97, p. 159 (1924).



the Douglas bag and the corrugated rubber tubing were carefully washed with oxygen mixture. Special attention was paid to any possible leakage round the mouth, since the breathing is so heavy that there would appear to be a risk of sucking air inwards.

A second possible difficulty, which can, however, be discounted, is due to the change in the nitrogen equilibrium of the body. Assume that the body weight of the subject is 50 kgm., and the water content of the body about 25 kgm. The solubility of nitrogen in water at 38° C. is 0.01280; therefore, when in equilibrium with air the body of this subject would hold in solution  $320 \times 4/5$  c.c., or 256 c.c. of nitrogen; in changing to a 50 per cent. oxygen mixture, out of 256 c.c. of nitrogen  $256 \times (0.8 - 0.5)$  c.c., or 76.8 c.c., can be turned out of the body. If the new equilibrium of nitrogen be attained in 3 minutes, the volume of nitrogen turned out in 1 minute is 26 c.c. This extra nitrogen from the body would cause a disturbance in the apparent oxygen intake of the order of 50 c.c. per minute, but as compared with the amounts actually obtained this is negligible. Thus the only serious error to be avoided is that due to possible leakage inwards of air.

The following table shows the oxygen intake in several subjects, every precaution being taken to avoid leakage inwards of, or contamination by, air:—

Speed : steps per minute.	Subject.	Fore- period of exercise - minutes.	Time of collection - minutes.	Ventila- tion. litres per minute.	Oxygen in inspired gas per cent.	Oxygen used per minute : c.c.	O <sub>2</sub> ex- pired per minute : c.c.
240	C.N.H.L.	2 0	1 5	102	59.10	3465	4008
244	C.N.H.L.	3 0	1 0	104	56.97	4025	4259
268	S.S.	2 5	1 0	115.5	57.58	5290	4500
280	S.S.	2 5	1 0	123	54.37	4827	4612
280	S.S.	3 0	1 0	115	55.17	5865	4278
244	K.F.	2 0	0 5	93.5	57.68	3460	3572
240	K.F.	1 5	0 5	91.2	57.68	3420	3748
240	K.F.	2 0	0 5	97.2	57.58	4179	3664
240	K.F.	2 0	0 5	111	52.92	3763	4140
240	K.F.	2 0	0 5	93.3	42.92	3554	3601
240	K.F.	2 0	0 5	101	54.82	4363	4140
240	K.F.	1.5	0 5	95	51.26	4161	3819
240	K.F.	1.5	0 5	86	53.00	3806	2354
240	K.F.	1 5	0 5	95	air	2565	3667
240	K.F.	2 0	0 5	89.5	air	2325	3470
240	K.F.	2 0	0 5	106	air	2856	3695
240	K.F.	1.5	0 5	108	air	2685	4244

In the case of the subject K.F., some of the maximal oxygen intakes when breathing air are added. The average value of the oxygen intake in this case, when breathing air, is 2606 c.c. per minute; when breathing a rich

oxygen mixture, while moving at the same speed under similar conditions, it is 3849 c.c., *i.e.*, there is an increase of 47 per cent. in the oxygen intake, when breathing about 50 per cent. oxygen. From the above results, the previous conclusions are clearly confirmed. It is striking that with K.F., moving at this speed, the total lung ventilation is nearly the same in air and in oxygen, as also is the amount of CO<sub>2</sub> expired per minute. It is noticeable throughout the table that the maximum of the CO<sub>2</sub>-output occurs, not with the maximum O<sub>2</sub>-intake, but with the maximum lung ventilation.

The highest value of the oxygen intake for K F. is (per kgm.) the greatest recorded hitherto. This subject weighs 50 kgm. so that per kgm. the maximum value is 87.3 c.c. per minute; his average value even is 77 c.c. per minute. For S.S. the maximum value is about 78 c.c. per minute per kgm.; for C.N.H.L. the greatest value recorded previously was 76.3 c.c. per kgm. per minute. These very high values appear only during very severe and rapid effort, while breathing a high percentage of oxygen. The greatest value ever previously recorded while breathing air was 57 c.c. per minute per kgm. (on A.V.H.); the maximal value on K.F. is the same (56.7 c.c. per kgm. per minute); his maximal value while breathing the oxygen mixture is just about 50 per cent. greater.

Since there is no reason for assuming an unsaturation of the arterial blood, while breathing air, sufficient to account for this large increase in the oxygen intake, we must attribute this rise of oxygen intake to an increase in the circulation rate, *i.e.*, presumably to the heart muscle itself; the heart must be able to change, by some unknown mechanism, its output per beat, in accordance with the degree of unsaturation of the arterial blood which reaches it through its coronary vessels.

#### *Summary.*

Certain technical errors are discussed which might conceivably affect the determination of the oxygen intake in man during severe exercise while breathing an oxygen mixture, and experiments were recorded in which every possible precaution was taken to avoid the incidence of such errors. These show, as before, that the maximum oxygen intake may be increased 50 per cent. by the breathing of a mixture rich in oxygen. This can be attributed only to an increased circulation rate of the blood.

In conclusion, it is a pleasure to acknowledge my indebtedness to Prof. A. V. Hill for his advice, and to Mr. C. N. H. Long and Mr. S. Scheinfein, who were subjects in some of the above experiments.

*Physiological and Anatomical Investigations on Mimosa pudica.*

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The plant is a multicellular organism, and hence necessity arises for inter-communication and inter-action between more or less distant organs. I have shown elsewhere (1) that this is accomplished in the plant, as in the animal, in two different ways, by translocation of matter and by transmission of motion. The first is secured by the *slow* movement of fluids carrying chemical substances in solution, such as occurs in the circulation of sap. The second is brought about by the *rapid* propagation of protoplasmic excitation, such as the nervous impulse in the animal. The evolution of a nervous system for the transmission of excitation in the living organism is a matter of much theoretical importance. It is therefore of interest to investigate the transmission of excitation in the undifferentiated protoplasm of the plant. In the course of my investigations on nervous reactions in plants during the last twenty years (2, 3, 4, 5) many important facts have been discovered which throw light on the more complex phenomena in the animal. A suitable subject for investigation is *Mimosa pudica*, in which the pulvinus acts as a motile effector, the excitation being conducted along definite channels.

It is unfortunate that in most investigations on the nature of the transmission of excitation in plants, hydro-mechanical disturbance was involved by the crude and drastic methods of stimulation employed, which led to erroneous conclusions. For the determination of the true character of the transmission, it is essential that the stimulus should be such as to produce no mechanical disturbance. The important characteristics of the impulse, moreover, can only be determined by accurate measurement of its normal velocity and the changes induced in it under physiological variations. It is therefore necessary to obtain, by means of an automatic recorder, reliable measurements of the period of transmission in the conducting tissue, as well as the latent period of the effector. These requirements are fulfilled by my Resonant Recorder (2), which enables time-intervals as short as 0.005 sec. to be determined with very great precision (3).

I have carried out a complete series of investigations on the transmission of excitation in *Mimosa*, from simple conduction to the highly complex trans-

formation of afferent to efferent impulse in the reflex arc. On account of limitations of space, description of the phenomenon of the reflex arc is for the present postponed. I propose to describe the results of the physiological and anatomical investigations as follows :—

A. Nature of the Transmission—

1. Polar Excitation by Constant Current.
2. Electrotonic Block arrests Transmission.
3. Velocity of Transmission.
4. Effect of Temperature and of Desiccation on Velocity.
5. "Bahnung" or Facilitation.
6. Theory of the Transpiration-Current in Conduction.
7. Supposed Transmission across a Water-gap.
8. Simultaneous Determination of Translocation of Stimulant and of Transmission of Excitation.
9. One-sided Propagation under Unilateral Stimulus.
10. Polar Action of Electric Current.
11. Forcing the "Block" by Stronger Stimulus.
12. Untenability of the Theory of the Transpiration-Current.

B. Anatomico-physiological Characteristics of Conducting Tissue—

13. Effects of Peripheral and Central Stimulation.
14. Electric Localisation of the Conducting Tissue in the Petiole.
15. Conducting Tissue in the Stem.
16. Phloem-Connection between Stem and Leaves.

C. Cause of Higher Sensitivity of Motor Organ—

17. Anatomy of Petiole-pulvinar Junction.
18. Excitable, Semi-excitable and Unexcitable Pulvini.
19. Protoplasmic Modification of Contractile Cells.
20. Micro-chemical Detection of "Active" Substance.

D. Summary.

A. NATURE OF THE TRANSMISSION.

1. *Polar Excitation by Constant Current.*

I have shown (2) that under the polar action of an electric current the undifferentiated protoplasm of the plant exhibits excitatory reactions similar to those given by animal tissues; that is to say, with moderate current, excitation takes place at the cathode at make; with stronger current, it occurs

both at cathode-make and at anode-break. These characteristic protoplasmic reactions are manifested not merely at the electrodes but also at a distance. The fact of the transmission of these excitations is a crucial proof that the transmission is due not to water-movement but to protoplasmic propagation. I have previously obtained such a transmission in the petiole of *Mimosa* (5), a similar result in the stem is given in Experiment 6.

### 2 *Electrotonic Block arrests Transmission.*

Further crucial evidence proving that the transmission of excitation in *Mimosa* is protoplasmic, is furnished by the observation that it is arrested during the application of an electrotonic block (5). No such arrest would be produced were the transmission due to water-movement.

Koketsu (6) has been successful in repeating some of my experiments in proof of the physiological character of excitatory transmission in *Mimosa*. He obtained the characteristic polar excitation at the make and break of a constant current, and also the arrest of conduction by the interposition of an electrotonic block.

### 3. *Velocity of Transmission.*

In summer, the velocity in thick petioles of *Mimosa* is 30 mm., while in thin petioles I find it to be as high as 350 mm. per second. The velocity in the stem is lower than in the petiole. In either case the velocity of the transmission of excitation is far greater than that of the transpiration-current.

### 4. *Effect of Temperature and of Desiccation on Velocity.*

As in the animal nerve, so also in the conducting tissue of the plant, rise of temperature enhances the rate of transmission. A rise of temperature of about 9° C., in the median range, nearly doubles the velocity in *Mimosa*. Artificial desiccation of the petiole by glycerine enhances the conducting power. The velocity of transmission is, in general, higher on a dry than on a damp day.

### 5. "*Bahnung*" or *Facilitation*.

In highly excitable specimens of *Mimosa*, strong or long continued stimulation induces a depression or fatigue in the rate of transmission. But in sub-tonic specimens, the after-effect of stimulus is to confer an enhanced power of conduction. The conducting path in such a case is canalised by stimulus.

The characteristic results described above prove conclusively that the transmission of excitation in the conducting tissue of the plant is of the same

character as that in the conducting nerve of the animal. The above experiments were carried out mostly with the petiole of *Mimosa*. I shall presently describe experiments which will show that the transmission in the stem also is of the same nature.

#### 6. *Theory of Transpiration-Current in Conduction of Excitation.*

A theory has recently been proposed that conduction in *Mimosa* is brought about by the transpiration-current in the xylem carrying some stimulating substance excreted by the wood in consequence of stimulation. In support of this theory Ricca (7) describes the following experiment. When a shoot of *Mimosa Spegazzinii* is cut completely across, and the two cut ends joined by a tube filled with water, then strong stimulation of the lower piece by a flame is often followed by the fall of leaves of the upper portion. The application of an extract obtained from the internodes, to the cut end of the stem, was also observed to cause excitation of the leaves. Snow (8) obtained similar results in *Mimosa pudica*.

In order to justify the transpiration-current theory, it is necessary to prove (1) that there is a transmission of stimulus across the water-gap; (2) that a hypothetical stimulant is excreted as the result of stimulation; and (3) that the velocity of the transpiration-current conveying the stimulant is the same as that of the excitatory impulse.

#### 7. *Supposed Transmission across a Water-gap.*

In experimenting on this subject, Koketsu (6) divided the petiole of *Mimosa* into two pieces, and connected them by a water-tight tube filled with water. Stimulus applied at the distal half was never found to be conducted across the water-gap. It has, however, been urged that the mechanism of transmission in the stem is very different from that in the petiole, a supposition which will be shown to be totally groundless.

My experiments with the stem on the supposed transmission of stimulus across a water-gap were carried out on no fewer than fifty different specimens of *Mimosa*, grown in a vigorous condition in the grounds of the Institute. I employed not one but diverse modes of stimulation. Some of the experiments were carried out with cut specimens: their excitability, I have shown (9), becomes fully restored in the course of a few hours.

*Experiment 1.*—The stems were cut under water, and the two cut ends, enclosed in a watertight tube, were brought in contact with each other. I first tried the effect of electric stimulation; the sensitiveness of the upper and

lower portions of the stem was such that separate application of induction shock of intensity 2, lasting for one second, caused the fall of the leaves on each piece. After recovery, stimulus was applied to the lower piece; the intensity of stimulus was increased to the maximum by pushing the secondary coil close to the primary; the duration of application was also increased from 1 to 20 seconds. In spite of this intense stimulation there was no evidence of conduction across the gap.

I next tried thermo-electric stimulation. A thin platinum wire was wound round the lower piece of stem. The passage of a strong electric current made the wire red-hot and scorched the stem. But even under this excessively strong stimulation the upper piece remained unexcited, showing that there was no transmission across the water-gap. The experiments described above were carried out repeatedly with numerous specimens. In not a single instance was there any evidence of transmission across the gap.

As regards the view that extracts of the stem cause stimulation, no such effect was observed, though the experiment was repeated with 20 different specimens. It is, however, conceivable that some vegetable extracts may act as stimulants. I found, for example, that various vegetable alkaloids of a poisonous nature caused excitation, when applied in minute doses to the cut end of the stem. But it would be unreasonable to conclude from this that alkaloid is excreted by the plant on the application of the minimal stimulus effective in inducing excitation.

One of the crucial experiments for determining the character of the impulse is the following. We apply a stimulant and find the rate at which it is translocated in the plant, either (1) by the use of a stain which is also a stimulant; or (2) by the employment of a colourless stimulating fluid, the extent of translocation of which with the movement of sap can be detected by the employment of a suitable "developer." The speed of the excitatory impulse induced by the stimulant can be determined from the fall of the leaves. The identity of the two speeds would serve to support the transpiration-current theory; the theory would be disproved if the two speeds were found to be widely different.

#### 8. *Simultaneous Determination of Translocation of Stimulant and of Transmission of Excitation.*

*Experiment 2.*—Application of the stain methylene blue in moderately strong solution causes stimulation. The thin and long flower-stalk of *Biophytum sensitivum* is very efficient in the transmission of excitation.

When the stalk is stimulated in any way, by mechanical or electric stimulus, or by a chemical irritant, the excitatory impulse travels downwards (against the normal direction of ascent of sap) and, reaching the rosette of leaves, causes the closure of the sensitive leaflets from the centre outwards. The top of the flower-stalk was cut across and a piece of moist cloth placed on the cut end. After the recovery of the leaflets from the excitatory impulse due to the cut-stimulus, methylene blue was applied at the cut end. The excitatory impulse caused by the stimulant was found to reach the innermost pair of the leaflets of the leaf after an interval of 30 seconds. The length of the flower-stalk and the stem through which the impulse was transmitted was 60 mm. The translocation of the staining solution downwards was determined by examining a longitudinal section of the flower-stalk under the microscope. The translocation after 30 seconds was found to be quite negligible, certainly not more than through 0.3 mm. This was due rather to slow diffusion than to movement of sap, which, normally speaking, is upwards and not downwards. Even on the assumption that the transpiration-current moved the sap downwards, the rate of transmission of excitation is 200 times quicker than the rate of sap-movement. I obtained similar results with eosin stain.

*Experiment 3.*—This was carried out with *Mimosa pudica*, the stimulant being dilute HCl. The extent of its translocation was ascertained by the application of  $\text{AgNO}_3$  solution, which produces a white precipitate. The acid was applied at the tip of the uppermost leaf. (It is a good plan to wash the tip previously with dilute ether, to promote absorption of the applied solution.) Half a dozen experiments gave very similar results. Of these the following may be taken as typical. The excitatory impulse initiated by the application of acid at the tip travelled downwards and caused successive closure of seven leaves in the course of 40 seconds, the distance of transmission being 2 cm. Examination of a section treated with  $\text{AgNO}_3$  solution showed that the acid had not been translocated downwards against the direction of the normal ascent, but had remained practically localised at the point of application.

The above results prove that under conditions where there is no translocation of the chemical stimulant by the movement of sap, there is still a fairly rapid propagation of the excitatory impulse. The transmission of excitation is therefore in no way connected with the movement of sap. These facts definitely disprove the theory of the co-operation of the transpiration-current in the conduction of excitation.



### 9. *One-sided Propagation of Unilateral Stimulus.*

*Experiment 4.*—I took a vigorous specimen of *Mimosa* growing in a pot, and stimulated the stem at a point between the second and third leaf counted from the top in the same vertical line which passed through them. Stimulation was effected by scratching the vascular bundle, taking care that the pin-point only grazed the surface of the bundle without reaching the wood. An excitatory impulse was nevertheless generated which caused the successive fall of leaves not only upwards but also downwards against the direction of the normal transpiration-current. This excitatory transmission in both directions at the same time occurred not only under mechanical but also under electric stimulation. In the latter case the plant was uninjured, the unilateral stimulation being effected by means of two electrodes applied on the epidermis. In the experiments just described, the excitatory transmission occurred only on the stimulated side of the stem, the leaves on the opposite side remaining unaffected. Explanation of this characteristic action will be given presently.

*Experiment 5.*—A very remarkable result is obtained, when the “scratch stimulus” is increased in effective intensity by repetition, or when the intensity of electric stimulation is increased. Under unilateral stimulation of greater intensity, the ascending impulse not only caused the fall of leaves from below upwards, but after reaching the apex it crossed over to the opposite side, on which the sequence of fall of leaves was from above downwards. The direction of propagation on one side was thus with the direction of the transpiration-current, while on the opposite side it was against that direction.

### 10. *Polar Action of Electric Current.*

*Experiment 6.*—The erect stem of *Mimosa* bears motile leaves in alternate series. If we number the leaves from below as 1, 2, 3, 4, 5, 6, 7 and 8, the odd numbers are vertically over each other to the right, the even numbers being to the left; the two rows are thus on opposite sides of the stem. Two electrolytic connections for passage of current from a battery were made on the left side, at points intermediate between the leaves 4 and 6. A fairly strong current was maintained by the application of 6 volts. On starting the current, excitation was induced at the cathode which was above, and the leaves 6 and 8 underwent a fall in serial succession. Owing to the electrotonic block, the excitation could not be transmitted downwards. When the current was broken, excitation was induced at the anode and the impulse was transmitted downwards, the leaves 4 and 2 falling one after the other (fig. 1).

In this physiological mode of causing excitation, no hydro-mechanical dis-

turbance is produced. There is no injury caused to the intact plant. The excitatory impulse initiated at the anode-break travelled against the direction

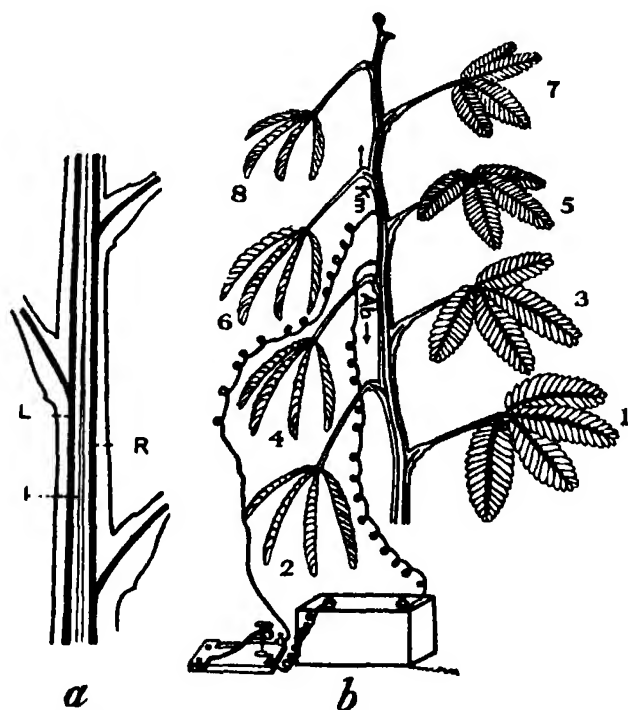


FIG. 1. (a) Diagrammatic representation of the course of the conducting tissue in the stem (b) Effect of polar excitation by constant current (See text)

of the transpiration-current. The characteristic excitations at cathode-make and at anode-break prove conclusively that the conduction of excitation is a phenomenon not of water-movement, but of protoplasmic propagation.

The physiological mechanism in the stem is, therefore, the same as that in the petiole, in which it is admitted that conduction takes place not by the movement of sap, but by excitatory impulse propagated along the phloem. Snow (8) is constrained to admit that the theory of the transpiration-current is inadequate "to cover all the phenomena of conduction in *Mimosa*, including conduction in the leaf, and the subordinate phenomenon of 'high-speed conduction' in the stem." In very excitable specimens of *Mimosa*, the ascending impulse due to cathode-make was found to be conducted to the apex and then to cross over, causing a descending impulse, as was found in Experiment 5.

*Explanation of the ascending and the descending impulse in stems.*—Examination of a vertical section which includes the odd and even series of leaves

(see fig. 7a) shows that there are two main conducting vascular strands situated on opposite sides of the stem. They are separated from each other throughout their length except at the apex where they meet. The left strand gives off branches to the even series and the right strand to the odd series of leaves (fig. 1a). Unilateral stimulus of moderate intensity applied on the left side, initiates an impulse which following the conducting channel reaches the apex; the impulse then descends by the conducting channel on the right. The ascending impulse on one side is thus reversed to a descending impulse on the opposite side.

It should be mentioned here that besides the two main strands in the stem there are intermediate ones; these latter are not continuous with, but contiguous to, the two principal strands. (See fig. 5, also fig. 1a.) There is thus a "block" or resistance to the spread of excitation in a lateral direction. Hence unilateral stimulus of moderate intensity is only conducted lengthwise (that is, along the line of least resistance) and not laterally in a direction across the stem.

#### 11. Forcing the "Block" by stronger Stimulus.

*Experiment 7.*—The resistance to the spread of excitation sideways may be overcome by a still stronger stimulus. Under the action of a more intense "scratch stimulus," or of an increased intensity of induction shock or stronger polar action of current, the hitherto unilateral conduction becomes outspread and the leaves on *both* sides of the stem exhibit the excitatory fall.

#### 12. Untenability of the Theory of the Transpiration-Current.

This theory, as already stated, assumes that conduction of excitation is dependent on (1) the excretion of a stimulating substance excreted on stimulation of the wood, and (2) on the transport of the stimulant by the transpiration-current in the vessels. But it has been shown that conduction takes place even when the wood remains free from stimulation. Further, the transpiration-current in intact specimens is normally upwards, but conduction takes place both upwards and downwards at the same time. Unilateral stimulus of moderate intensity gives rise to an impulse which is conducted only on the stimulated side. A stronger stimulus causes an impulse which ascends to the apex and, after crossing over, descends on the diametrically opposite side. These characteristic results cannot be explained on the transpiration-current theory. A fully satisfactory explanation is, however, afforded by the fact that the two strands of physiologically conducting tissue meet at the apex. Simultaneous determination of the extent of transport of chemical stimulant, and of the distance of pro-

pagation of excitation, showed that while the stimulant had remained practically localised at the point of application, the propagated excitation had travelled through a considerable distance. The conduction is therefore in no way connected with the movement of sap. These facts, individually and collectively, disprove the theory of the transpiration-current. The characteristic protoplasmic excitation at cathode-make and at anode-break, and the subsequent propagation of excitation to a distance, prove, on the other hand, that the conduction in the petiole and in the stem of *Mimosa* is a phenomenon of protoplasmic transmission.

#### B. ANATOMICO-PHYSIOLOGICAL INVESTIGATION OF THE CONDUCTING TISSUE.

It is now admitted that in the petiole conduction is effected by the propagation of protoplasmic excitation along the phloem and not by any sap-movement. Before dealing with the question of the distribution of the conducting tissue in the stem, it is desirable to localise the conducting strands in the leaf, and to trace the nervous connection between the central pulvinus with the peripheral sub-petioles carrying the sensitive leaflets.

##### 13. *Response to Peripheral and Central Stimulation.*

I have shown elsewhere (10) that the pulvinus of *Mimosa* is a highly complex organ whose four quadrants act as four distinct effectors, each determining its characteristic responsive movement. The petiole contains four main vascular bundles, as shown in the transverse section (fig. 2), in which E is the epidermis, C the cortical tissue, S the cylinder of sclerenchyma for mechanical strength and protection of the conducting tissue in the interior. F is one of the four vascular bundles which contain the conducting phloem; O is the central pith. The four nerve-ends appear to coalesce in the pulvinus to form an almost continuous ring, in spite of which they are functionally distinct. Since in the conduction of excitation, the phloem-strands in each bundle function as does a nerve in the animal, I designate each conducting strand as a plant-nerve; each of these connects a particular quadrant of the pulvinus at the centre, with the corresponding sub-petiole at the periphery (fig. 3).

The continuity of the conducting channel between the pulvinus and the periphery through the phloem can be made out by anatomical dissection: it can also be independently traced by two different methods of response. In the first, stimulus is applied at the periphery, and the effect of the centripetal impulse observed in the characteristic response caused by one or other of the

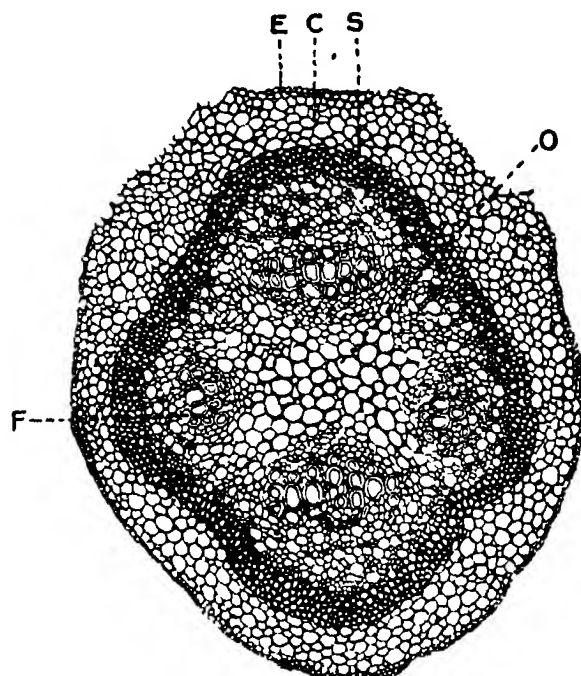


FIG. 2.—Transverse section of petiole of *Mimosa* showing four main bundles enclosed in a protecting cylinder of sclerenchyma (two stray bundles at upper corners not shown). E, epidermis ; C, cortex , S, sclerenchyma ; F, one of the four main vascular bundles , O, pith.

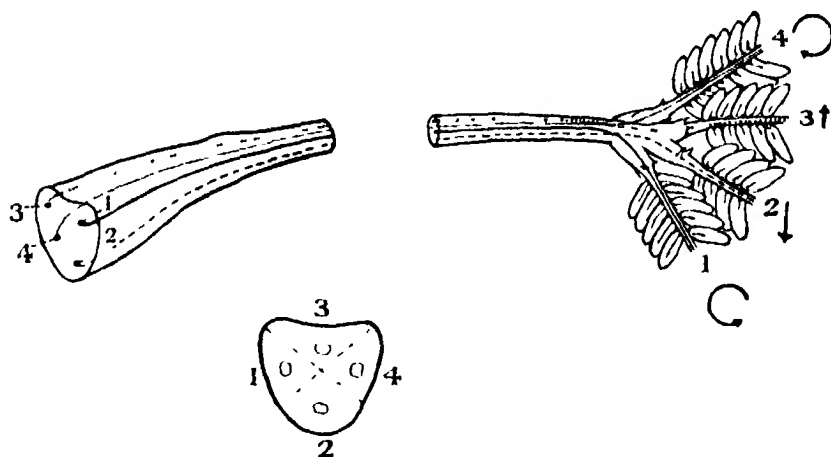


FIG. 3.—Diagrammatic representation of nervous communication between the four quadrants of the pulvinus and the four sub-petioles. Lower figure shows the four quadrants. Direct stimulation of quadrant (1) or indirect stimulation of sub-petiole (1) induces a left-handed torsional movement. The effects of stimulation of other quadrants and sub-petioles are represented by a straight or a curved arrow. (See text.)

four quadrants. In the second method, we stimulate successively the four nerve-ends in the pulvinus and observe the response of each of the sub-petioles as it responds to the resulting centrifugal impulse.

*Peripheral Stimulation.*—Employing the stimulus of light, direct stimulation of quadrant (1), or indirect stimulation of the leaflets of sub-petiole (1), induces a similar responsive movement, a left-handed torsion of the leaf. Hence this quadrant must be in nervous communication with the sub-petiole (1). The effects of direct or indirect stimulation of the other quadrants are diagrammatically represented in the figure. It is by the particular innervation of the motor organ that the leaf undergoes the purposeful movements by which it places itself at right angles to incident light so as to absorb the largest amount of radiant energy (10).

*Central Stimulation.*—Though the nervous connection between the periphery and the pulvinus is proved by the response of the leaf to the peripheral stimulation of the sub-petioles, yet the converse experiment of stimulating the nerve-ends in the pulvinus is of great interest. The central end of each nerve is stimulated by thrusting a sharp-pointed pin into one of the four quadrants till the nerve-end is reached. After a little practice it is easy to feel the moment when the pin touches the vascular bundle containing the conducting tissue.

*Experiment 8.*—When the nerve-end in quadrant (1) is cautiously stimulated, an excitatory impulse is generated which, travelling outwards, causes response only of leaflets on sub-petiole (1). Stimulation of the nerve in the lower quadrant (2) causes response in sub-petiole (2). Similarly, stimulation of the nerve-ends in quadrants (3) and (4) elicits corresponding response in sub-petioles (3) and (4) respectively. The effects of stimulation at the central end demonstrate once more the definite nerve-connection between the centre and the periphery.

#### 14. *Electric Localisation of the Conducting Tissue in the Petiole.*

I succeeded in localising the conducting nervous tissue by the electric method. No visible change occurs in a nervous tissue during the passage of excitation: the only means of its detection is in the electric change of galvanometric negativity exhibited by the tissue during the passage of excitation. By the employment of this electric method (4) I was able eighteen years ago to prove that it is the phloem that conducts excitation in all plants. In regard to nerve and nervous impulse I quote the following from Bayliss (11):—

“The effect of anything happening at one end is conveyed with great

rapidity to the other end of the nerve, wherever it may be. Nerve fibres have no other function than that of carrying excitation. . . . It is usual to speak of a 'propagated disturbance' passing along the nerve, or sometimes 'nervous impulse.' The most sensitive apparatus has been able to detect with certainty one kind of change accompanying the passage of the propagated disturbance, namely, an electric effect." The conducting tissue of the plant exhibits all the characteristics of a nerve described above, and there is therefore justification for describing it as plant-nerve.

I localised the conducting nerve in the interior of the petiole of *Mimosa* by means of the electric probe. Galvanometric negativity was only detected when the probe came in contact with the phloem (see fig. 4a); neither the xylem nor the pith showed any excitatory electric reaction. The electric excitation was found to exhibit two maxima; one inside and the other outside the xylem. This led to the discovery (4) of two conducting strands; an outer, which is the usual phloem; an inner, which I term the inner phloem (fig. 4).

*Demonstration of two phloems by double straining.*—The existence of a double phloem in the vascular bundle of *Mimosa* was so unexpected that I wished

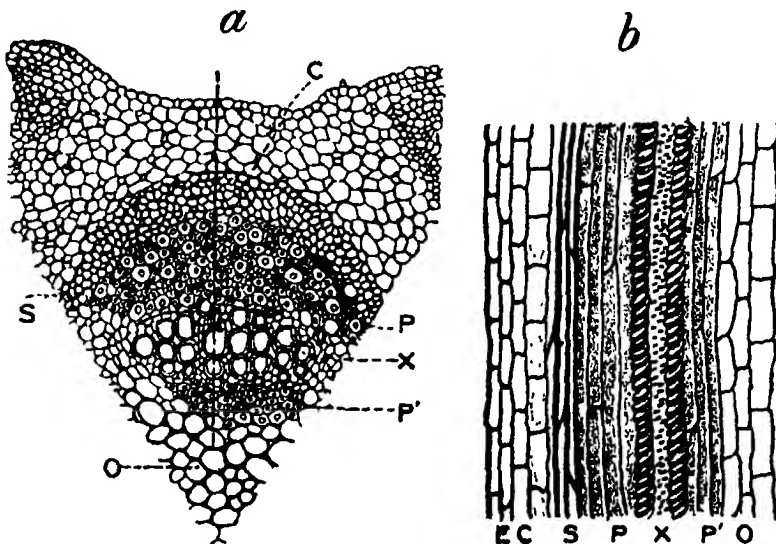


FIG. 4.—Petiole of *Mimosa pudica*. Transverse and longitudinal sections of a single vascular bundle. *a*, Transverse section; the line of passage of electric probe shown dotted. C, cortex; S, sclerenchyma; P, external phloem; X, xylem; P', internal phloem; O, pith. *b*, Longitudinal section of the bundle; note elongated tubular cells, both in the external and in the internal phloem. (The section passed through one side of the bundle and not through the middle.)

to verify it by other independent methods. The first of these is the employment of stains which differentiate the phloem from the xylem. Delafield's hæmatoxylin and safranin stain the phloem a rich violet, while the xylem is stained deep red. Hæmatoxylin and Bismarck brown produce even a stronger contrast, the lignified cells and vessels being stained brown while the phloem is stained violet. Finally light green and safranin are regarded as very good stains: the phloem is stained green in sharp contrast to the xylem, which is stained red. These staining reagents clearly show that there are two phloems in the bundle, one outside and the other inside the xylem.

What is the anatomical characteristic of the phloem which renders it such a good conductor of excitation? The phloem of *Mimosa* consists mainly of elongated tubular cells; it has been pointed out by Haberlandt that elongation of cells in the direction of propagation, and the consequent decrease in the number of transverse septa, increases the velocity of propagation. In addition to the tubular cells there may be sieve-tubes in which protoplasmic continuity exists between neighbouring cells through minute perforations in the dividing septa. I find that the number of sieve-tubes in the phloem is not very large; the conducting elements must therefore consist principally of elongated tubular cells arranged in longitudinal series. In the analogous case of nerve-fibres in animals, conduction is not necessarily dependent on a continuity of the protoplasm through the entire length of the conducting path, but on the close contiguity of the ends of the fibres. It is believed on good grounds that there is no protoplasmic continuity where neurone joins neurone, the separating membrane being known as the synapse, which, acting as a valve, allows excitation to be more easily conducted in the one direction than in the other.

The fact that close contiguity is also effective in conduction across septa was established in Experiment 7, where excitation under strong stimulus was conducted laterally from one conducting strand to the next. The proof of the existence of synapsoidal membranes (transverse septa interposed between the conducting tubular cells) is found in the fact that there is a preferential direction of conductivity in the conducting tissue of *Mimosa*, as in the nerve of the animal.

#### 15. *Conducting Tissue in the Stem.*

Some of the upholders of the transpiration-current theory insist that the phenomenon of conduction in the stem is of a different character to that in



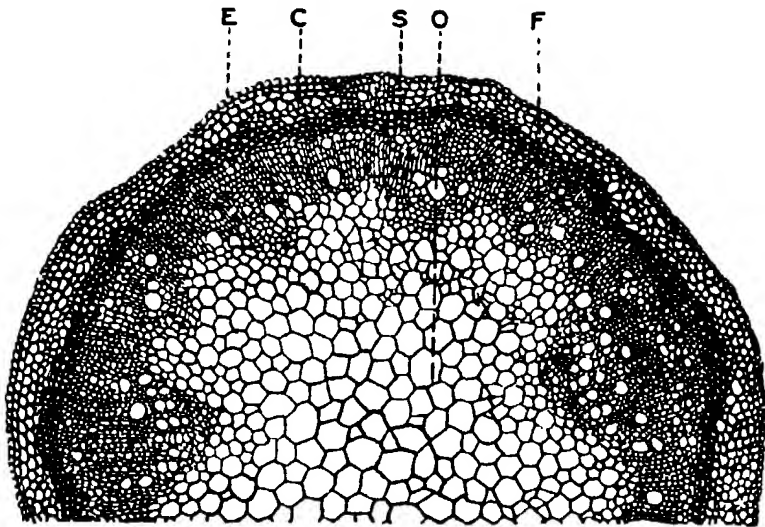


FIG. 5.—Transverse section of the stem of *Mimosa pudica* (one-half shown in the figure).

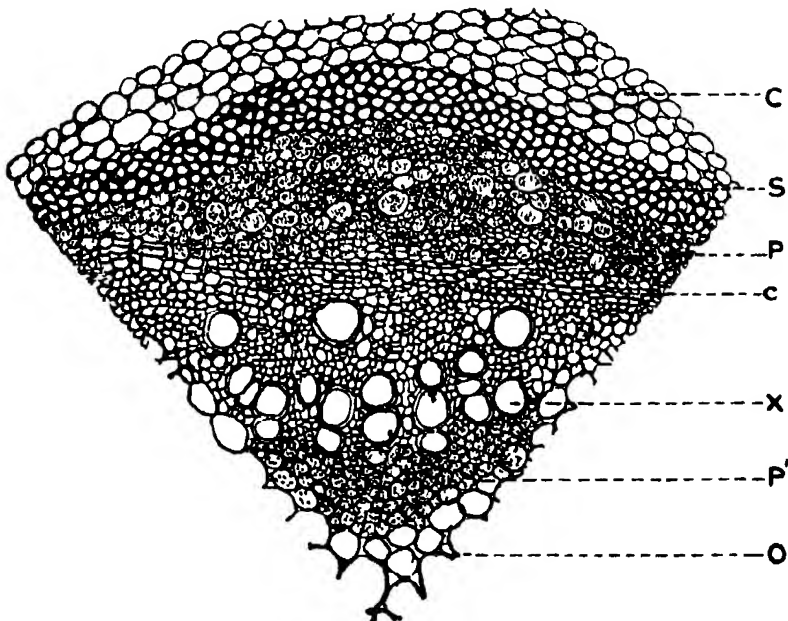


FIG. 6.—Transverse section of single bundle in stem. C, cortex; S, sclerenchyma; P, external phloem; c, layers of cambium; X, xylem; P', internal phloem; O, pith.

the petiole; there is, however, no justification for this assertion. Physiologically the conduction has been shown to be similar in the two organs

(Experiments 3 and 6). The anatomical structure of the conducting tissue is the same in both. Fig. 5 shows the circular arrangement of about a dozen bundles in the stem, each bundle containing the conducting tissue being laterally contiguous to two neighbouring ones. There is thus a partial block to the passage of excitation in a lateral direction, the conduction being normally up or down along the phloem-strands. It is only under stronger stimulus that the block is forced and the excitation is outspread in a lateral direction (see under 12). Fig. 6 is a reproduction from a photo-micrograph of the transverse section of a single bundle. The arrangement of different tissues here is the same as in the petiole. There are two phloems, external and internal, the xylem being interposed between the two.

#### 16. *Phloem-Connection between Stem and Leaves.*

It was stated that there are two opposite main bundles in the stem, from which lateral branches are alternately given off to the odd and even series of leaves. A longitudinal section of a young stem, which also passes through opposite leaves (fig. 7a), shows this very clearly. Treated with hæmatoxylin and safranin, the two opposite phloem-strands, stained deep violet, stand out most distinctly against the background of other tissues. They give off lateral branches to the leaves and their upper terminations meet at the apex. The establishment of this histological continuity accounts for the single impulse which ascends under moderate unilateral stimulation, it is also clear how under stronger unilateral stimulation the ascending impulse can cross over at the apex and become reversed into a descending impulse on the opposite side (Experiments 4 and 5).

#### C. CAUSE OF HIGHER SENSITIVITY OF MOTOR ORGAN.

Before dealing with the important question of the cause of the high sensitivity of the pulvinus, I briefly describe the anatomical features of this organ.

#### 17. *Anatomy of Petiole-pulvinar Junction.*

A longitudinal section of petiole and pulvinus reproduced from a photo-micrograph is given in fig. 7b. The section passes through the upper and lower bundles; these are separate in the petiole, but converge and meet in the pulvinus; the pith becomes thereby reduced, the cortex being increased proportionately. A very noticeable fact (which comes out under microscopic examination after staining) is that the cylinder of protective sclerenchyma is lignified only in the petiole; the lignification ceases as soon as the tissue

enters the pulvinus. The reason of this is that the tissue in the pulvinus must be pliable to allow rapid movement of the pulvinus under excitation.

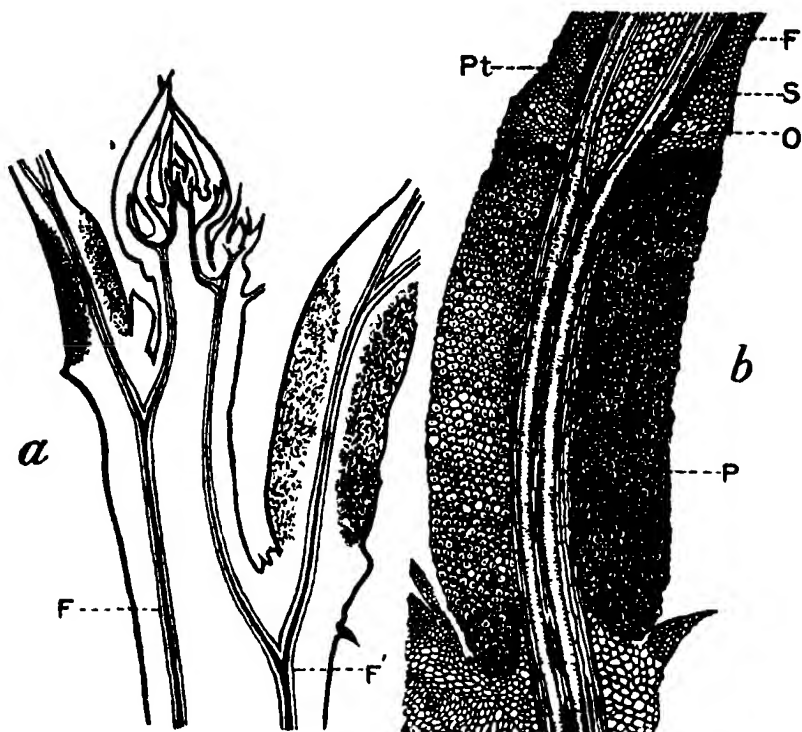


FIG. 7.—*Mimosa pudica* *a*. Longitudinal section of stem bearing leaves. The two ascending vascular bundles F, F' containing conducting tissue give off lateral branches to leaves, and meet at apex. Pulvinus of leaves represented dotted. *b*. Longitudinal section of petiole and pulvinus passing through upper and lower vascular bundles. F, the vascular bundle which meets its fellow in the pulvinus: S, protective sclerenchyma cylinder, lignification of which occurs only in petiole: O, pith disappearing at pulvinus: Pt, petiole the cells of which remain unstained; P, cells of pulvinus deeply stained; outline of contractile cells most sharply defined (See text)

The line of junction between the petiole and the pulvinus cannot be clearly detected, even under the microscope, for the cells of the petiole pass imperceptibly into those of the pulvinus. I have, however, succeeded in determining the exact line of demarcation by methods to be presently described.

#### 18. *Excitable, Semi-excitable, and Unexcitable Pulvini.*

The pulvini of leguminous plants all exhibit slow nyctitropic movements; but it is only a few, like the main pulvinus of *Mimosa*, that exhibit rapid and

almost instantaneous movement under even the feeblest stimulus. Such a pulvinus I distinguish as *excitable*. At the opposite extreme of sensitivity are the *unexcitable* pulvini of plants like *Phaseolus* or *Erythrina indica*, which under moderate or feeble stimulus exhibit no movement. Intermediate between the two extremes are the pulvinus of *Neptunia* and the secondary pulvini of *Mimosa* and *Neptunia*. These will be designated as semi-excitable. The difference of sensitivity between excitable and semi-excitable will be found from the following data. The maximum fall of the leaf of *Mimosa pudica* occurs within a single second of the reception of external shock: but the maximum fall of the leaf of *Neptunia* is not attained till after 180 seconds. *Neptunia* is therefore far less excitable and much more sluggish than *Mimosa*. The following are the examples of the three types of pulvini:—

*Excitable*.—Main pulvinus of *Mimosa*.

*Semi-excitable*.—Main pulvinus of *Neptunia*, and secondary pulvini of *Mimosa* and *Neptunia*.

*Unexcitable*.—Main pulvini of *Phaseolus* and *Erythrina*.

The question now arises what is the cause of this difference in excitability? Could we devise tests to detect the presence of some unknown “active” substance which confers high sensitivity?

#### 19. *Protoplasmic Modification and Exact Localisation of Contractile Cells.*

It appeared probable that the high excitability of *Mimosa* might be due to some protoplasmic modification and to the presence of an active substance in the contractile cells of the pulvinus. If so, the difference between the active and inactive cells might be brought out by the action of suitable chemical developers.

Thus, while hæmatoxylin and safranin stain the ordinary protoplasmic contents of inactive cells a faint violet, the protoplasmic modifications such as chromosomes nucleoli and centrosomes are stained deep red by the same reagents. It is true that they also stain lignified structures; but we are for the present concerned not with the staining of different tissue-systems, but the differential staining of the protoplasmic contents of active and inactive cells.

*Hæmatoxylin and Safranin Developer. Experiment 9.*—The application of the double stain to a longitudinal section of the pulvinus of *Mimosa* produced the most remarkable effect. It appeared as if a hand had with utmost care picked out every contractile cell and painted it deep red. The cells of the petiole remained practically unstained or stained faint violet. The line of

demarcation between the active cells of the pulvinus and the inactive cells of the petiole was very sharply defined. The extent lengthwise of stained active cells in the lower half of the pulvinus is slightly greater than in the upper half. There was an abrupt end of staining at the basal end, marked here by a minute prickle. The stained protoplasmic masses in the lower half appeared to be somewhat more dense than in the upper. The mass in each active cell is very distinctly differentiated under high magnification; the protoplasm of the neighbouring inactive cells of the petiole is not stained at all (fig. 8a).

*Safranin and light-green Developer.* *Experiment 10.*—This also produced a very great contrast between the active and inactive cells. The active cells were stained deep crimson, while the inactive cells were stained green. I found several other double stains which also demonstrated the protoplasmic modification in the active cells.

*Difference between excitable, semi-excitable and unexcitable Pulvini.*—The fact that no such protoplasmic modification exists in inactive cells of the pulvinus is clearly seen in the longitudinal section of the pulvinus of *Phaseolus* (fig. 8c)

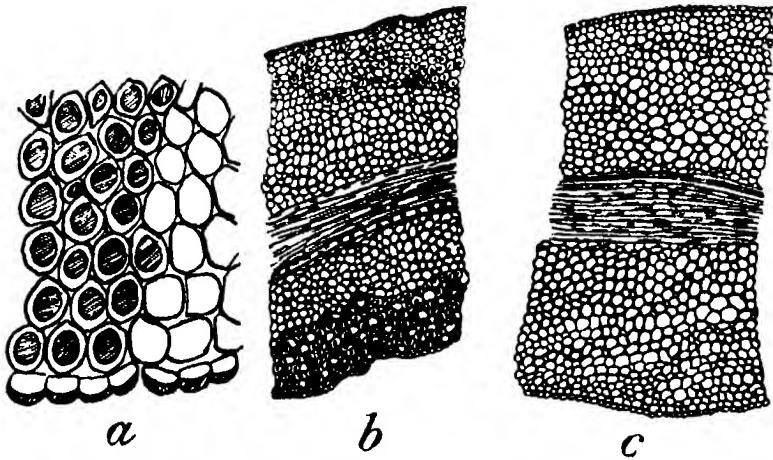


FIG. 8.—Longitudinal sections of different pulvini. (a) Excitable; (b) semi-excitable, and (c) unexcitable

a. Highly magnified pulvinar and petiolar cells of *Mimosa*. Protoplasmic cell-contents in contractile cells deeply stained; no such staining in petiolar cells.

b. Semi-excitable pulvinus of *Neptunia*. The area of stained active cells relatively less, with patches of inactive cells.

c. Unexcitable pulvinus of *Phaseolus*; cells all unstained.

in which not a single cell of the pulvinus was stained red on treatment with hæmatoxylin and safranin. Similar results were also obtained with the unexcitable pulvinus of *Erythrina*.

Again, the intensity and extent of staining is found to give a measure of the excitability of the motor organ. Fig. 8b represents the effect of staining on a longitudinal section of the semi-excitabile pulvinus of *Neptunia*. In *Mimosa* the staining of the cells extended right up to the vascular strand, but *Neptunia* exhibits a significant difference. The staining in the lower half of the pulvinus extended barely to one-half the thickness, there are, moreover, patches of cells which remained unstained. In the upper half of the pulvinus the extent of staining is less than a third of the depth; even in this third, large patches were not stained. The secondary pulvini of *Mimosa* and *Neptunia* showed similar characteristics.

The facts given above prove (1) that there is a protoplasmic modification in the active cells, such modification being detected by differential staining; (2) that this staining differentiates the active and the inactive pulvinus, (3) that the line of demarcation between active and inactive cells in a petiole-pulvinus preparation becomes sharply defined by the differential action of the staining agents, and (4) that the relative extent and density of staining affords a measure of the excitability of the motor organ.

We have next to consider the presence and nature of the substance which confers such a high degree of activity on the cells for the performance of mechanical work in the rapid movement of the leaf.

## 20. *Micro-chemical Detection of the "Active" Substance*

The energy for rapid movement of the leaf must ultimately be derived from oxidation of some active substance present in the cells of the excitable pulvinus. The substance must be highly oxidisable, so that it can absorb oxygen from any available source, it should therefore be capable of reducing oxides.

*Application of Osmic Acid. Experiment 11* --This oxidising agent was rapidly reduced by the protoplasmic contents of the active cells into a lower oxide, which produced a dark stain. The contrast between the active and inactive cells under osmic acid is as strongly marked as that produced by the double-stains. The dark staining could still be obtained after treatment of the sections with cold and warm alcohol or with ether. The active substance cannot therefore be fat or lipid, for the cells still contained a powerful reducing group characterised by double or triple bonds. This is justified by the fact that no staining was obtained after bromination, which converts the unsaturated into a saturated compound.

## SUMMARY.

Experimental results of a crucial character are given which show that the theory of the transpiration-current, as the agent of conduction of excitation in *Mimosa*, is entirely opposed to facts. The theory assumes that conduction is brought about by the transpiration-current in the xylem, carrying some stimulating substance excreted in consequence of the stimulation of the wood. But it has been shown that conduction takes place even when the wood remains entirely unstimulated. Again, the transpiration-current in intact specimens is normally upwards, but it has been shown that conduction of excitation takes place both upwards and downwards at the same time. On application of a chemical stimulant to the tip of the leaf of *Mimosa*, simultaneous determinations of the transport of the stimulant, and of the excitatory impulse generated by it, prove that while the stimulant remained localised at the point of application, the excitatory impulse had travelled through a considerable distance: the conduction is therefore in no way connected with the movement of sap. The characteristic protoplasmic excitation at cathode-make and at anode-break, and the subsequent propagation of excitation to a distance, prove, on the other hand, that conduction in the petiole and in the stem of *Mimosa* is a phenomenon of transmitted protoplasmic excitation.

The nervous character of the conduction is further proved by the following facts. It is arrested during the maintenance of an electrotonic block. Rise of temperature enhances, and fall of temperature lowers its velocity, which is nearly doubled by a rise of about 9° C. Facilitation is often produced by previous stimulation: stimulus thus canalises its own conducting path. The velocity of conduction in thick petioles of *Mimosa* in summer is about 30 mm. ; in thin petioles it is often as high as 350 mm. per second ; it is of a higher order than the velocity of the transpiration-current.

By means of the electric probe the conducting or nervous tissue in *Mimosa* has been localised in the phloem of the vascular bundles. The conducting tissue is characterised by elongated tubular cells arranged in longitudinal series. Microscopic examination shows that the anatomical structure of the conducting tissue in the stem is similar to that in the petiole.

The distribution of the phloem in the stem, and the continuity of the conducting tissue between stem and leaves, have been very clearly made out by the use of double-staining. The two main longitudinal conducting strands, situated diametrically opposite to each other, meet at the apex. They give off lateral branches to the odd and even series of leaves. Diverse effects of

conducted excitation in the stem follow from the characteristic distribution of the nervous tissue. Local stimulation at any point (in the vertical line passing through the one row of leaves) gives rise to two impulses which travel in opposite directions at the same time, causing fall of leaves both above and below the point of stimulation. The ascending impulse due to unilateral stimulation of moderate intensity is conducted only along the stimulated side, the impulse generated by a stronger stimulus is transmitted further, it crosses over at the apex, and the ascending impulse at one side becomes reversed into a descending impulse at the opposite side. These characteristic effects are also strongly against the transpiration-current theory.

The petiole contains four main conducting phloem-strands, which meet and form an almost continuous ring at the central pulvinar end. These four conducting strands terminate separately in the four sub-petioles carrying the sensitive leaflets. The nervous connection between centre and periphery has been traced both anatomically and physiologically. Peripheral stimulation of each of the four sub-petioles gives rise to characteristic responsive movements of the leaf, up or down, a left-handed or a right-handed torsion. It is by the particular innervation of the motor organ that the leaf undergoes the purposeful movements by which it places itself at right angles to the incident light so as to absorb the largest amount of radiant energy. The nervous connection between the centre and periphery is independently demonstrated by observing the effect of the centrifugal impulse produced by successive and separate stimulations of the different nerve-ends in the pulvinus, which cause the responsive fall of the sensitive leaflets of the sub-petiole in connection with each stimulated nerve-end.

The high motor excitability of the pulvinus of *Mimosa* is shown to be due to special protoplasmic modification of the active cells. By the application of differential stains the line of demarcation between the contractile and non-contractile cells at the petiole-pulvinar junction has been distinctly made out. It is further shown that the relative extent and density of staining affords a measure of the excitability of the motor organ. The characteristic staining is absent in inactive pulvini such as those of *Phaseolus* and of *Erythrina*.

The energy of rapid movement must ultimately be derived from oxidation of an active substance present in the protoplasmic contents of cells of excitable pulvini. It is shown that they contain an active substance which is highly oxidisable and reactive, being an unsaturated compound with double or treble bond combinations.



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*On Cytochrome, a Respiratory Pigment, Common to Animals,  
Yeast, and Higher Plants.*

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*Introduction.*

Under the names myohæmatin and histohæmatin MacMunn (1884-1886) described a respiratory pigment, which he found in muscles and other tissues of representatives of almost all the orders of the animal kingdom. He found that this pigment, in the reduced state, gives a characteristic spectrum, with four absorption bands occupying the following positions: 615-593/567.5-561/554.5-546/532-511/. When oxidized, the pigment does not show absorption bands. In 1887 MacMunn described a method by which it can be extracted in a "modified form" from the muscles of birds and mammals. He found the pectoral muscle of a pigeon to be the most suitable material for

the extraction of "myohæmatin," in the belief that it was the sole colouring matter of the muscles in a pigeon bled to death. From this "modified myohæmatin" he also obtained other derivatives, such as acid hæmatin and hæmatoporphyrin, and he finally arrived at the conclusion that myo- and histohæmatin are respiratory pigments different and independent from hæmoglobin and its derivatives.

In 1889 Levy carefully repeated MacMunn's experiments in extracting myohæmatin from muscles of birds and mammals and obtained the substance described by MacMunn as "modified myohæmatin." But he regarded this substance as an ordinary hæmochromogen, derived from hæmoglobin.

Levy's paper was soon followed by a reply from MacMunn (1889), and by a discussion between this author and Hoppe-Seyler (1890), who fully supported Levy and refused to take into consideration the presence of myohæmatin in invertebrates devoid of hæmoglobin. As to the four-banded absorption spectrum of myohæmatin, which, according to MacMunn, can be seen in a fresh muscle of mammal or bird, Hoppe-Seyler explained it as a mere superposition of bands of oxyhæmoglobin on the surface of the muscle with the bands of reduced hæmoglobin of the deeper layer, and possibly also with the bands of a small amount of hæmochromogen. Hoppe-Seyler finally dealt with the CO compound which he had obtained from MacMunn's "modified myohæmatin" present in the extracted fluid. This compound, which is in all respects similar to the compound obtained from ordinary hæmochromogen, Hoppe-Seyler brings as conclusive evidence against the existence of myohæmatin as a separate respiratory pigment.

In 1890 MacMunn tried once more to defend his position, but his defence was not even replied to by Hoppe-Seyler, who merely appended to MacMunn's paper a short editorial note, stating that he considered all further discussion as superfluous, MacMunn not having brought any fresh evidence in support of his views. Hoppe-Seyler's note ended the discussion and MacMunn's new respiratory pigment was gradually forgotten. The term myohæmatin still made occasional appearances in the literature, but authors mentioning it have seldom seen the pigment or even read MacMunn's original papers, those who have seen the pigment have misunderstood its properties, and have not failed to show that they were aware of Hoppe-Seyler's criticisms, with which they were in full agreement.

*Methods.*—For the spectroscopic examination of living organisms, cells, tissues or their extracts, two instruments have been used—the microspectroscopic ocular of Zeiss and the Hartridge reversion spectroscope. Zeiss's

microspectroscope was mainly used for the detection of the pigment when its concentration was very low, and for the examination of opaque tissues, portions of organism, suspensions of cells or turbid fluids. It was also used for examination of rapid oxidation and reduction of the pigment in cells or complete living organisms. For the precise determination of the position of absorption bands, the Hartridge reversion spectroscope gave exceptionally good results. It was, however, slightly modified by Dr. Hartridge for use with the microscope. The main modification consisted in inserting in front of the slit a double-image Wollaston prism \*. Calibration of this instrument was obtained by a determination of 14 sharp lines of emission spectra, giving a straight line when plotted. A strong source of light such as the Nernst lamp was used with both microspectroscopes.

### *Distribution of Cytochrome.*

In the course of my study on the respiration of parasitic insects and worms, I have found that the pigment myo- or histohæmatin not only exists, but has much wider distribution and importance than was ever anticipated even by MacMunn. Considering that this pigment is not confined to muscles and tissues, but exists also in unicellular organisms, and further, that there is no evidence that it is a simple hæmatin in the proper sense of the term, the names myo- and histohæmatin, given to it by MacMunn, are misleading. In fact, as we shall see later, there is ample evidence that this pigment is not a simple compound, but a complex formed of three distinct hæmochromogen compounds, the nature of which is not yet completely elucidated. I propose therefore to describe it under the name of *Cytochrome*, signifying merely "cellular pigment," pending the time when its composition shall have been properly determined. This name, which expresses also its intracellular nature, does not, however, relegate the pigment to any definite compound, an important consideration inasmuch as the properties of various compounds cannot hereafter be ascribed to it without good evidence.

I have found cytochrome in the cells and tissues of a great number of individuals of the following groups and species of animals: - Turbellaria: *Dendrocæla lactea*; Oligochætes: *Allolobophora chlorotica*, *Helodrilus caliginosus*; Nematodes: *Ascaris megalocephala*, *Ascaris suis*; Molluscs: *Limnaea peregra*, *L. stagnalis*, *Helix nemoralis*, *H. aspersa*; Crustacea: *Oniscus* sp., *Asellus aquaticus*, *Cancer pagurus*; Myriapods: *Lithobius forficatus*, *Geophilus* sp.;

\* A description of a new reversion microspectroscope will shortly be given by Dr. Hartridge in the 'Journal of the Royal Microscopical Society.'

Arachnids: *Epeira diademata*. Most of the orders of insects, 40 species of which have been examined. Amongst insects the following is a list of common species, which can be easily recognised and examined:—Diptera *Musca domestica* (ordinary house fly), *Homalomyia canicularis* (lesser house fly), *Calliphora erythrocephala* (blow fly), *Sarcophaga carnaria*, *Glossina palpalis* (tsetse fly), *Gastrophilus intestinalis*, *Eristalis tenax*, *Anopheles maculipennis* and *Culex pipiens*; Hymenoptera: *Vespa crabro* (hornet), *V. vulgaris* (wasp), *Bombus terrestris* (bumble bee), *Apis mellifica* (honey bee), Coleoptera: *Carabus* sp., *Melolontha vulgaris*, *Tenebrio molitor*, *Dytiscus marginalis*, Lepidoptera: *Pieris brassicae*, *Bombyx mori* (silk-worm moth), *Galleria mellonella* (wax moth), Hemiptera: *Notonecta glauca*, *Corixa* sp.; Orthoptera: *Forficula auricularia*, *Blatta orientalis*.

The study of this pigment in vertebrates required more complicated manipulation, such as perfusion of their circulatory system, and was therefore confined to a few examples: frogs, pigeons, guinea-pigs and rabbits.

The number and wide range of systematic distribution of the species which show this pigment clearly, and which have been enumerated either by MacMunn or myself, is so great that it may safely be concluded that cytochrome is one of the most widely distributed respiratory pigments. Moreover, cytochrome is not confined to animal cells alone. I have found it, and in great concentration, in cells of bacteria, those of ordinary bakers' yeast, and also in some of the cells of higher plants. To avoid all confusion in the terms which will be used in this paper it is important to mention beforehand that cytochrome (= myohæmatin = histohæmatin) is a pigment distinct both from blood hæmoglobin and from muscle-hæmoglobin (= myochrome of Morner = myoglobin of Günther) or their derivatives. In many cells cytochrome may, however, coexist with hæmoglobin.

#### *General Characters of Absorption Spectrum of Reduced Cytochrome.*

*Cytochrome in Animal Tissues.*—The best material for the study of the absorption spectrum of cytochrome is provided by the thoracic muscles of the honey bee. Specimens of bees frozen at  $-7^{\circ}\text{C}$ . are allowed rapidly to thaw. The head and abdomen are cut off, and by compressing the thorax laterally with the fingers the thoracic muscles are expelled in one mass through the anterior opening of the thorax. The muscles of 2 or 3 bees, compressed between a slide and coverslip and examined with the Zeiss microspectroscope, show clearly a very characteristic absorption spectrum (fig. 1) composed of four bands (a, b, c, d), the position of which can be determined only with the Hartridge-

microspectroscope For each band I have taken an average of 10 readings and although the pigment was examined *in situ*, the variations between individual

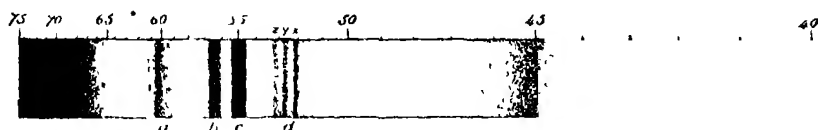


FIG. 1 — Absorption spectrum of cytochrome in thoracic muscles of a bee

readings were only about 7 Ångström units. The position of maximum intensity of the bands in the bees is as follows: —a, 6046; b, 5665; c, 5502; d, 5210. The relative width and intensity of the bands, in other words, the general aspect of the spectrum, varies naturally with the thickness of the layer of tissue examined. In moderate concentration the relative width of the band is approximately as follows — (in  $\mu\mu$ ): a, = 614–593, b = 567–561, c = 554–546, d = 531–513. Fig. 1 gives an idea of the absorption spectrum of cytochrome in a layer of muscle 0.65 mm. thick, examined with the Zeiss microspectroscope. It shows that band a is very asymmetrical, being darker near the border turned towards the red end of the spectrum, band b is symmetrical, but in bees is much lighter than the band c, the latter being the strongest band in the spectrum; band d which is faint and wide is also asymmetrical, being darker near its short wave end, it also shows a lighter space near the middle, giving to the whole band the appearance of being composed of at least two distinct bands (x and y) corresponding respectively to 5210 and 5280. In transparent muscles rich in cytochrome, a third very fine band (z) could be seen near the green end of the band d (532).

The absorption spectrum of cytochrome in other organisms differs very little from that of the honey-bee (fig. 2). In the thoracic muscles of a female bumble bee, the position of all the four bands is exactly the same as in a honey bee, band a only appearing to be wider and more prominent. In the thoracic muscles of a wax moth (*Galleria mellonella*) the position of the bands is: a, 6046; b, 5657; c, 5495; d, 5200; but their relative intensity is the same as in bees. In the thoracic muscle of *Dytiscus marginalis* the bands correspond to: a, 6038; b, 5664; c, 5495; d, 5205. In the pharyngeal or radula muscles of *Helix aspersa* the bands are not so easily measured, and band b is of approximately the same intensity as band c, while band d is hardly perceptible. Their positions are: a, 6035; b, 5650; c, 5495; d, 5200 (?). The positions of the bands in the heart muscle of a frog are: a, 6040; b, 5660; c, 5500; d, 5205. The cytochrome in the heart and other muscles of a guinea-pig shows the

bands as follows : - *a*, 6045 ; *b*, 5662 ; *c*, 5500 , *d*, 5205. In other animals, such as earthworms, crustacea (especially the heart), myriapods (muscles),

610	605	600	595	590	585	580	575	570	565	560	555	550	545	540	535	530	525	520		
6046	a Bee wing muscles								5655	b				5502	c				5205	d
6038	Dytiscus . .								5661					5505					5203	
6046	Galleria . .								5651					5503					5200	
6035	Helix radula .								5650					5500					5200	
6045	Frog heart muscle								5662					5500					5205	
6045	Guinea pig "								5662					5500					5205	
6035	a Yeast cells								5645	b				5490	c				5190	d

FIG 2---Positions of the four main absorption bands of cytochrome (*a*, *b*, *c*, and *d*) in various organisms

various insects, different organs of molluscs and birds (pectoral muscles of pigeon), cytochrome was always found with its four characteristic bands, and the variation in the position of these bands was not greater than that which was already shown by the examples mentioned above. It is impossible at this stage to decide whether the small differences observed in the position of the bands of cytochrome in different organisms correspond to a real difference in the composition of this pigment, or are only of the nature of an experimental error, caused by the presence of small amounts of other pigments, such as lipochromes or hæmoglobin.

*Cytochrome in yeast cells.*—A slightly wetted fragment of bakers' yeast, compressed between two slides to the thickness of 0.6 mm, and examined with the Zeiss' microspectroscope, shows very clearly the characteristic spectrum of cytochrome, with its four bands. *a*, *b*, *c*, *d*, very similar to those of the thoracic muscles of a bee. Owing to the greater opacity of yeast, the correct position of the four bands is more difficult to determine. This is mainly due to the loss of light which is unavoidable in the Hartridge reversion spectroscope. The insertion of a Ramsden ocular within the tube of the microscope increases the amount of light and makes it possible to obtain a fairly accurate reading. The positions of the four bands in yeast obtained by this method are. *a*, 6035, *b*, 5645 ; *c*, 5490 ; *d*, 5190. The positions of these bands in yeast, compared with those of bees or other animals, are slightly nearer the blue end of the spectrum. These differences in the position of the bands are, however, of a much smaller magnitude than those which are known to occur between

hæmoglobins of various origins.\* We can say, therefore, that cytochrome, in spite of its great range of distribution, shows a very characteristic, easily recognisable and uniform absorption spectrum. This uniformity of the spectrum indicates, moreover, the great similarity in the chemical composition and the properties of this pigment whatever may be its origin.

The four absorption bands of cytochrome are of unequal intensity: thus band *c* is usually the strongest, then come *a* and *b*, and finally *d*, which is faint, and, when the concentration of pigment is low, may easily be overlooked. It is important to note, that this relative intensity of the four bands, although very general, is not so constant as the position of the bands. In some tissues bands *b* or *a* are almost as strong as the band *c*.

When a tissue showing clearly all the four bands is gradually compressed between two slides, the bands become faint and disappear in inverse order to their intensity. During this process the tissue shows a succession of spectra with 3, 2, or 1 absorption bands. This observation shows that the reduction in number of the bands of cytochrome which may be found in tissues of different organisms denotes only a lower concentration of the pigment. Moreover, in the great majority of those cases, the remaining absorption bands can be detected on increasing the depth of tissue examined. This naturally applies to tissues devoid of all pigments other than cytochrome.

#### *The Relative Concentration of Cytochrome in Different Tissues and Cells.*

The relative concentration of the pigment in the tissue is shown by the relative depth of the tissue necessary for the microspectroscope to reveal a clear absorption spectrum with four characteristic bands. A more precise indication is obtained by the thickness of the compressed tissue at which the band *c* is extinguished. The concentration of cytochrome varies not only with the species examined but also, and to a much greater extent, in different tissues of the same animal. In parasitic worms, such as *Ascaris lumbricoides*, the highest concentration is found in the spermatozoa and eggs. In a mollusc, *Helix aspersa*, the order of concentration is: (1) pharyngeal or radula muscles; (2) the walls of the stomach; (3) the genital glands and other organs. In crabs the muscles of the heart contain much more cytochrome than the strong muscles of the legs. In insects the thoracic muscles are the richest in this pigment, but in small concentration it is found also in other organs: intestinal wall, brain,

\* The difference in the position of  $\alpha$ -band of  $\text{HbO}_2$  in *Planorbis* and *Chironomus* is about 31 Å. and that between *Planorbis* and the muscle-hæmoglobin of a mammal may reach as much as 64 Å.

genital glands. In frogs the order of concentration is (1) heart, (2) testes, (3) walls of the stomach; (4) muscles of the body; (5) other organs. In mammals the greatest amount of pigment is found in the heart muscle, and then in the muscles concerned with mastication, the diaphragm and the muscles of the legs.

For the study of cytochrome we shall select the muscles of insects and yeast in which it is unmixed with other pigments and in sufficient concentration.

#### *Oxidized and Reduced Cytochrome.*

The absorption spectrum with four characteristic bands corresponds to the reduced state of cytochrome, while the spectrum of the pigment in its oxidized state, at least in the concentration found in the tissues, shows no distinct absorption bands, but only a very faint shading extending between 520-540 and 550-570  $\mu$ . The oxidation and reduction of the pigment can be easily observed in yeast. If a shallow tube (30 mm. high) is half-filled with a suspension of bakers' yeast in water (20 per cent.), and the suspension then examined with the Zeiss microspectroscope, the four absorption bands may be clearly seen; but when the air is rapidly bubbled through the suspension the cytochrome becomes oxidized and the bands disappear. If the current of air is stopped the pigment becomes reduced and the four bands rapidly reappear.

A similar result can be obtained by shaking a 5 c.c. yeast-emulsion in a test-tube and examining it with the microspectroscope. When, instead of air, a current of  $N_2$  is passed through the yeast emulsion, or when the latter is shaken with  $N_2$ , the cytochrome remains in a reduced state, showing all the time its characteristic four absorption bands. Similar results are obtained with the thoracic muscles of bees or the striated muscles of a guinea-pig. But in these cases the oxidation and reduction are better seen in the broken-up muscles, which may be spread on a slide. When the muscle is exposed to air the cytochrome is seen in its oxidized form, but when the slide is covered with another slide and the space around the muscle filled with glycerin, the cytochrome becomes reduced and the four bands appear. In these experiments, cytochrome is oxidized by  $O_2$  of the air and reduced by the tissue itself. The oxidation of a reduced cytochrome can easily be obtained without shaking with air, by adding to the tissue a small quantity of potassium ferricyanide or of  $H_2O_2$ . On the other hand, when the reducing power of the tissue has been destroyed, cytochrome, which then becomes easily oxidized with air, can be readily reduced by adding to the tissue a small quantity of a reducer such as  $Na_2S_2O_4$ .

The conditions which determine the oxidation and reduction of cytochrome are not equally influenced by the change of temperature. The oxidation



depends upon the rate of diffusion of oxygen into the suspension of yeast on the tissue and is but little affected by temperature; its reduction is due to the chemical activity of the cells which contain the pigment, and therefore has a high temperature coefficient, *e.g.* --(a) At the ordinary room temperature ( $18^{\circ}$ - $20^{\circ}$ ) the oxidized cytochrome of yeast usually becomes reduced by the activity of the cells in six to eight seconds. When the oxidized yeast is kept at  $0^{\circ}$  to  $-2^{\circ}$  C. complete reduction takes place in 70 seconds to 2 minutes. (b) Again, in yeast emulsion kept in a thin layer in a Petri dish at  $-2^{\circ}$  to  $-4^{\circ}$  C. cytochrome usually remains oxidized. Similar results have been obtained with the thoracic muscles of blow-flies and those of bees. These tests show that a low temperature inhibits the reducing power of the tissue more easily than oxidation power of cytochrome.

#### *Action of Narcotics on Cytochrome.*

When a drop of weak solution of KCN is added to the suspension of yeast, no matter how actively this suspension is shaken with air or pure  $O_2$ , the cytochrome remains completely reduced, and continues to show the characteristic bands, which do not differ in the slightest degree from the bands of an ordinary reduced cytochrome. The concentration of KCN which stops oxidation of cytochrome is about  $n/10,000$ , and a much lower concentration, such as  $n/100,000$ , inhibits to a great degree the oxidation power of the pigment.

Further and more important, when a drop of KCN is added to the suspension of yeast, kept at a low temperature and previously oxidized by a current of air, the cytochrome becomes immediately reduced, just as if KCN was acting as a powerful reducing agent. In fact, KCN does not act as a reducer, but inhibits the oxidation of cytochrome, while it does not inhibit other oxidation processes which may accompany the reduction of our pigment.

The action of sodium pyrophosphate is similar to that of KCN. It also inhibits the oxidation of cytochrome only, while it does not arrest the reduction of this pigment. Other substances, such as formaldehyde, ethyl alcohol, acetone, and ethyl urethane, act in a very different way. All these substances, even in concentrations which kill the cells of yeast, do not inhibit the oxidation of cytochrome. On the contrary, in such a concentration they completely stop the reduction of cytochrome, which then remains oxidized indefinitely.\*

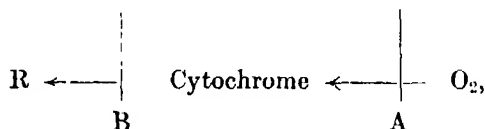
\* The oxidation of cytochrome in this case differs slightly from ordinary oxidation. In ordinary oxidation all the four bands fade away more or less simultaneously. In presence of urethane or formaldehyde band *c* disappears the first, while the other three bands remain and band *b* seems to be even intensified. On shaking the emulsion for a long time with air, all the bands disappear, band *b* being the last to go.

In lower concentrations they delay the reduction of oxidized cytochrome, though they do not completely stop it.

If a suspension of yeast in ethyl urethane is shaken with air until the cytochrome becomes completely oxidized, and KCN solution is then added to this suspension, the cytochrome does not become reduced. It remains oxidized, because the reducing action of the cells is inhibited, or even destroyed, by urethane, while KCN has no effect on oxidized cytochrome. When the reducing power of the cells is not completely destroyed by the action of urethane, on adding KCN to such suspension a gradual but slow reduction of cytochrome can be observed.

The facts given above show clearly that in relation to the oxidation process in cells in which cytochrome is involved all the inhibitors of oxidation can be separated into two distinct categories, the actions of which are fundamentally different. To one category belong KCN and sodium pyrophosphate, to the other such substances as alcohols, urethane, and aldehydes. The first category (A) inhibits the oxidation of cytochrome, the second (B) inhibits its reduction.

Diagrammatically this can be represented in the following way:—



R being the substances which reduce the oxidized cytochrome, B and A indicating the places of rupture in the oxidation system produced respectively by the substances of the corresponding category of inhibitors. This seems to indicate that, at least for oxidation systems similar to that of cytochrome, the problem of the action of narcotics needs further investigation and existing theories require careful revision.

#### *The Natural Behaviour of Cytochrome in Living Organisms.*

We have previously seen that in a compressed fragment of bakers' yeast or in an emulsion of yeast, cytochrome is usually found in a reduced state. Yet we can hardly speak of natural conditions of life in the case of yeast, which is an organism highly modified by long and constant selection. We have found, however, that while in bakers' yeast the concentration of cytochrome is very high, in brewers' yeast it is very low. This is undoubtedly correlated with the different modes of life of these two categories of yeasts.

In the dissected muscles of a perfused guinea-pig, cytochrome is usually

found in a completely, or almost completely oxidized form, and the reduction takes place when the muscles are excluded from the air, *e.g.*, pressed between two slides. The thoracic muscles of insects, on the other hand, even when they are rapidly taken out from the body and immediately examined, almost always show the reduced form of the pigment.

In both these cases we are dealing with portions of tissues excised from an organism and therefore not examined in natural conditions. To understand the function of this pigment it is important to find out in what state this pigment is present in a normal living organism. The main difficulty in answering this question consisted in finding suitable material for such an investigation. One insect, however, the common wax-moth (*Galleria mellonella*) answered the purpose. Several active specimens of this moth were selected out of a large stock bred in the Laboratory, and the thorax of each was carefully cleaned from scales. These specimens were then attached by the ventral surface to a slide (by means of small droplets of gum arabic) and the thorax carefully examined with Zeiss microspectroscope and a strong light.\*

The following are the results of these observations :—

- (1) Female of *Galleria* remained very quiet, except for the occasional expulsion of an egg and a somewhat rhythmic movement with ovipositor. The thorax being of a yellow colour showed better the long-wave portion of the spectrum, but no absorption band could be detected
- (2) In specimens of males and females which began to struggle constantly, vibrating their wings in efforts to detach themselves from the slides, the bands of cytochrome gradually appeared, band *a* being very clear, and bands *b* and *c* appeared as almost fused into one band.
- (3) When these specimens ceased to move and stopped the vibration of the wings, the bands became very faint and hardly detectable.
- (4) In a specimen which showed no absorption bands (the cytochrome being oxidized) a slight pressure exercised upon the thorax made the bands of a reduced cytochrome to appear.
- (5) Specimens of *Galleria* fixed to the glass bottom of a special air-tight gas-chamber and examined spectroscopically showed no absorption bands. When  $N_2$  or coal gas was passed through the chamber all the four bands of reduced cytochrome appeared very rapidly. When  $N_2$  was cleared with air, the bands rapidly disappeared

\* In this condition the insects remained alive for long periods, and even after three hours the females, in spite of being attached to the slide, went on ovipositing.

- (6) When a specimen with oxidized cytochrome was exposed for a few seconds to vapours of KCN all the four bands rapidly appeared and the insect became motionless.
- (7) The same specimen being brought back into fresh air, the absorption bands of reduced cytochrome gradually faded away and the insect began to show signs of life.

The absorption bands of cytochrome shown by *Galleria* after exertion, still less after intense vibration with the wings, are never so strong as they appear in specimens exposed to pure  $N_2$  or to the vapours of KCN. This fact indicates that in natural conditions cytochrome is in the oxidized form, and that during exertion, however great, cytochrome becomes only partially reduced.

The above experiments with *Galleria* and the previous observations on yeast show that cytochrome acts as a respiratory catalyst, which is functional in oxidized as well as in a partially reduced form. The oxygen is constantly taken up by this pigment and given up to the cells. In the living organism the state of the cytochrome as seen spectroscopically denotes only the difference between the rates of its oxidation and reduction.

These experiments indicate also the rapidity of gas diffusion through the tracheo-spiracular system of an insect. It must be remembered that in winged insects the tracheal system of the thorax is highly developed, the trachea arising from the thoracic spiracles give off numerous branches, which on reaching the thoracic muscles give rise to innumerable small capillary tracheoles. These tracheoles penetrate into the muscular fibres, forming there a rich net of even more minute tracheoles. Ramón y Cajal (1890), who has carefully investigated the final branches of this system, has described them as being from  $0.1\mu$  to  $0.2\mu$  in diameter. The respiratory movements of insects, when they exist, ventilate only the large trachea, or air sacs connected with it, while the small trachea, to effect the gas-exchange, are dependent on pure diffusion. We can, therefore, easily explain the observations No. 4: the pressure exerted upon the thorax compresses the large trachea, and, therefore, partially cuts off the supply of fresh  $O_2$ ; this causes partial reduction of the cytochrome and the appearance of the absorption bands.

#### *Concentration of Cytochrome in Relation to Muscular Activity in Insects.*

We have previously seen, that among all the organisms examined the highest concentration of cytochrome is found in the thoracic muscles of flying insects. This has undoubtedly a connection with the peculiar activity of these muscles.

We know in fact from old experiments by Marey (1874) that the wing muscles of insects are capable of producing very rapid contractions. This author found that in the house-fly the wing makes 330 complete vibrations per second. The data given for other insects are: drone fly, 240 vibrations; bee, 190; wasp, 110; hawk moth, 72; dragon-fly, 28; and butterfly, 9. It was also proved that in a fly 330-340 electric shocks per second are required to produce the tetanic contraction of the wing muscle, which shows that the fly's muscle is capable of producing more than 300 separate contractions per second.

This peculiar property of insect-muscle explains the presence in the fibrils of so high a concentration of cytochrome. Moreover, careful examination of wingless insects corroborates this supposition, *e.g.*, the thoracic muscles of the wingless sheep-keds (*Melophagus ovinus*) hardly show the presence of cytochrome. The same applies to the thoracic muscles of the ordinary body louse (*Pediculus humanus*) or bed bug (*Cimex lectularius*). The best example is, however, shown by the winter moth (*Cheimatobia brumata*). The male of this moth, which is provided with well-developed wings and flies well, shows the presence of cytochrome clearly, while the female with reduced non-functional wings scarcely shows the pigment in the muscles. Finally, in cockroaches (*Stylopyga orientalis*) which do not fly, but are good runners, cytochrome is especially localised in the muscles of the legs.

Insects also furnish good material for the study of this pigment during their different stages of development. In the common blowfly cytochrome is already present in the eggs of this fly and in the muscles of the larva. The concentration of the pigment in these muscles is found to be approximately 12 to 15 times lower than that of the thoracic muscles of the adult fly. During the metamorphosis the concentration of cytochrome in the freshly-formed thoracic muscles of the pupa increases with its development. The adult insect, however, does not contain the maximum amount of the pigment immediately after hatching, this is reached during the life of imago, and undoubtedly depends upon the amount and composition of the food supply.\*

#### *Derivatives of Cytochrome and Nature of this Pigment.*

Several attempts have been made to extract this pigment in unmodified state from cells of yeast and the thoracic muscles of bees, where this pigment is the only colouring substance seen spectroscopically. Up to the present, however,

\* The fat body of these larvæ contains on the other hand a hæmochromogen-like complex showing three absorption bands, the position of which being approximately 603/558/524.

all attempts have completely failed. The pigments extracted showed marked differences from cytochrome both in the absorption bands and in other properties and can only be considered as its derivatives. I have succeeded, nevertheless, in securing ample evidence as to the family of respiratory pigments to which cytochrome belongs. The first important indication as to its nature is revealed by the hæmochromogen derivatives which have been obtained from this pigment. It is now certain (as we shall presently see) that the hæmochromogen compound obtained by H. Fisher and his co-workers from yeast is not derived from hæmoglobin (which they suspected to be present in yeast) but from cytochrome. It is also certain that the porphyrins extracted by these authors from yeast do not exist as such in yeast cells, but appeared also as derivatives of cytochrome

#### *Hæmochromogen Derivatives of Cytochrome.*

I *Hæmochromogen in Water Extract of Yeast*.—Dried yeast powdered with sand and extracted with water gives an opalescent yellow fluid which shows two absorption bands. One band ( $\alpha$ ) is very intense, narrow, much resembling the band *c* of cytochrome and occupying almost the same position ( $= 5485$ ); the other ( $\beta$ ) is faint, situated in the region of the band *d* of cytochrome and seems to correspond to the portion *x* of that band, its position is approximately  $521\mu\mu$ . This pigment does not combine either with  $O_2$  or with CO in neutral or acid solution; neither acid nor alkali shifts the position of its bands, but when KOH is added to the solution the pigment obtained can be oxidized with air and combines loosely with CO. The derivative thus obtained is therefore a hæmochromogen, which we will call hæmochromogen C. It is precipitated from the solution by alcohol,  $HgCl_2$  and heat. The

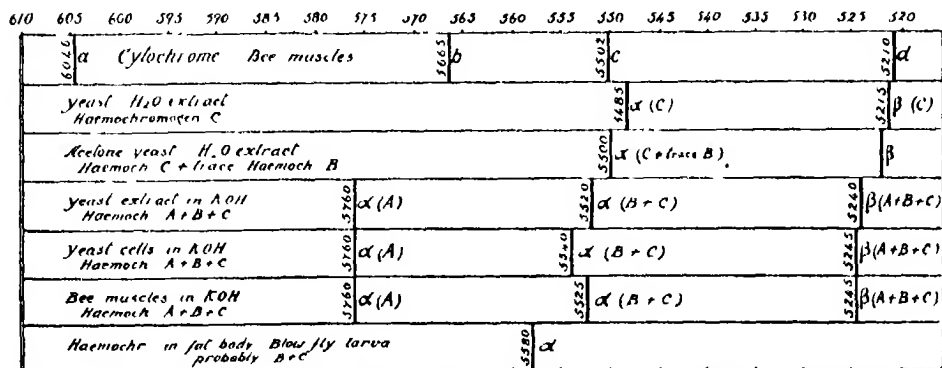


FIG. 3.—Positions of the absorption bands in cytochrome and in its three hæmochromogen derivatives, A, B, and C.

remaining yeast pulp still shows the presence of cytochrome, denoting that the extraction was only partial.

II. *Hæmochromogen in Water or KOH Extract of Acetone Yeast*—Yeast was thoroughly shaken with acetone and allowed to stand for 48 hours, filtered, washed with fresh acetone and dried. The fine powder thus obtained, when wetted again, does not show any band, but on addition of  $\text{Na}_2\text{S}_2\text{O}_4$  two bands appear, occupying the following positions: band  $\alpha$ -5500, band  $\beta$ -5220. The main band ( $\alpha$ ) is asymmetrical, its darkest portion corresponding to 5490, in other words, to the band *c* of cytochrome, while the side turned towards the red end of the spectrum is fainter, and this gives the impression of a strong band *c* being fringed with another band of lower intensity.

When acetone powder of yeast is treated with KOH and  $\text{Na}_2\text{S}_2\text{O}_4$  is added, the same two bands appear, but the pigment now combines with  $\text{O}_2$  and CO. This derivative is therefore also a hæmochromogen, though slightly different from the previous one.

The acetone solution separated from yeast is transparent and yellowish, and on spectroscopic examination in a tube of 10 cm. long shows faint bands resembling those of a porphyrin.

III. *Hæmochromogen in KOH Extract of Bees' Muscles and Yeast*—In strong solution of KOH both bees' muscles and yeast rapidly change their colour, yeast becomes distinctly orange, while the muscles turn orange-red. Spectroscopic examination of bees' muscles reveals a marked change in the position of the bands of cytochrome, band *a*, which has become fainter, is moved towards the blue end of the spectrum and stops at 5760, bands *b* and *c* seem to fuse together, forming a wide and strong band with its axis at 5525, while band *d* has become slightly stronger, though it remains broad and lies at 5245.

When yeast is shaken with strong solution of KOH and allowed to stand, the clear solution becomes separated from the jelly-like mass of yeast cells. The latter, after 24 to 48 hours' soaking in KOH, shows three bands occupying the following positions: 5760/5540/5245. The yellow fluid covering the yeast has similar bands, the positions of which are 5760/5520/5240/. When this fluid is shaken with air it becomes oxidised and the absorption bands disappear. On adding  $\text{Na}_2\text{S}_2\text{O}_4$ , all the bands reappear. When a current of coal-gas or CO is passed through this solution the bands disappear, seeming to be replaced by very faint shading, corresponding approximately to 565 and 528. The three previous bands do not reappear, however, on adding a reducing substance. By passing through this solution a very strong current

of air the CO becomes replaced by O<sub>2</sub> and on adding Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, the three bands reappear.

It is obvious, therefore, that the three-banded spectrum in KOH extract of yeast (or bees' muscles) belongs to a substance which has also the properties of a hæmochromogen compound, although it differs markedly from the two previously-described hæmochromogens.

*Composition of Cytochrome.*

The question arises now as to how a pigment such as cytochrome can yield hæmochromogens so spectroscopically distinct, and as to what is the nature of the three-banded hæmochromogen obtained in the KOH extract. The only plausible answer to both these questions is the assumption that cytochrome is composed of three components (*a'*, *b'* *c'*). Each of these components (*a'*, *b'*, *c'*) resembles spectroscopically a hæmochromogen in showing two absorption bands ( $\alpha$  and  $\beta$ ) in the reduced form and very faint bands or none in the oxidized form. The bands *a*, *b* and *c* of cytochrome would correspond to the  $\alpha$ -bands of these compounds, while the *x*, *y* and *z* of the band *d* would answer to their  $\beta$ -bands (fig 4). These three components (*a'*, *b'*, *c'*) may

	$\alpha$	$b$	$c$	$\overbrace{\alpha}^d$ $\overbrace{z\ y\ x}$
Cytochrome	$\alpha_1$	$\alpha_2$	$\alpha_3$	$\beta_1\ \beta_2\ \beta_3$
Compound $\alpha'$	$\alpha_1$			$\beta_1$
" $b'$		$\alpha_2$		$\beta_2$
" $c'$			$\alpha_3$	$\beta_3$
	Red			Blue

FIG 4 —Diagram showing the three hæmochromogen components *a'*, *b'* and *c'*, of cytochrome.

undergo some modifications which spectroscopically are manifested by the changes in the positions of their bands, and these modified components of the cytochrome in alkaline solution behave as hæmochromogens (A, B and C), each showing a two-banded spectrum in the reduced form (with a strong  $\alpha$ -band) and combining both with O<sub>2</sub> and CO. We can see now that the water extract of ordinary yeast contained only the hæmochromogen C, while the extract of acetone yeast, in addition to hæmochromogen C, contained a very small



quantity of hæmochromogen B, which shifted the middle of the  $\alpha$ -band towards the red end of the spectrum, and made this band asymmetrical.\*

The KOH extract of yeast contains all the three hæmochromogens (A, B, and C). The band lying at 5760 is the  $\alpha$ -band of hæmochromogen A,† and the wide strong band with axis at 5520 (or 5540 in KOH yeast pulp) corresponds to the two  $\alpha$ -bands of mixed hæmochromogen compounds B and C fused together. As to the  $\beta$ -bands of these three hæmochromogens, they are fused into one broad band with the axis at 5240 (or 5245).

It is important to note that when CO-hæmochromogen in KOH extract is aerated in presence of a reducer, the  $\alpha$ -band of hæmochromogen C appears first, occupying the position of the band *c* of cytochrome. This shows that in the KOH extract of yeast, just as in watery extracts of yeast or acetone-yeast, the position of the  $\alpha$ -band of the *c'* component of cytochrome remains always the same. It also shows that in the KOH extract the component *b'* of cytochrome undergoes some change, giving rise to hæmochromogen B, the  $\alpha$ -band of which, being nearer to that of C, mixes with it. We can also see that hæmochromogen B has greater affinity for CO than has hæmochromogen C.

The position of the  $\alpha$ -band of hæmochromogen A at 576  $\mu\mu$  being far in the long-wave part of the spectrum has nothing exceptional in it; in fact, this band lies exactly in the middle between the initial and final positions of the axis of the  $\alpha$ -hæmochromogen band of chlorocruorin, which according to Fox (1924) moves during preparation from 584  $\mu\mu$  to 569  $\mu\mu$ .

The supposition that cytochrome is composed of three distinct hæmochromogen-like compounds (*a'*, *b'*, *c'*) is supported by the following considerations .—

- (1) As we have seen previously the band *d* of cytochrome is not simple but is composed of three narrow bands, *x*, *y* and *z*. The band *d* seems thus to contain the three  $\beta$ -bands of three hæmochromogens.
- (2) Although the positions of the bands *a*, *b*, *c* and *d* of cytochrome are fairly uniform, the relative intensity of the bands varies with the organism

\* It is known now that different hæmochromogens may exist showing different absorption spectra, and that some of them do not combine with CO in neutral solution, but will combine when pH is changed. For the detailed discussion of this and other problems concerning hæmochromogen the reader is referred to the paper by Anson and Mirsky, which will shortly appear in the 'Journal of Physiology.' The porphyrin found in acetone fluid was probably derived partly from hæmochromogen *a'* and partly from *b'*.

† The evidence as to the hæmochromogen-nature of this compound is limited at present to the existence of two-banded absorption spectrum in the reduced state and to its O<sub>2</sub> and possibly CO compounds.

examined. Thus the band *b*, which in insects is much lighter than the band *c*, is in yeast almost as strong as *c*, and in snails there is no difference between their intensity. The band *a* may also vary in intensity, independently from the other bands. This variation in the relative intensity of the bands indicates that if the pigment is composed of three distinct compounds, the relative proportion of these compounds may vary to some extent.

- (3) When the suspension of yeast is normally oxidized the four absorption bands disappear almost simultaneously. When, on the other hand, the suspension of yeast is treated with alcohol, urethane or formaldehyde, and then shaken with air, the band *c* disappears rapidly, while the band *b* seems to become even more intense and remains for some time together with the band *a* and at least a part of the band *d*. After a longer or shorter interval the band *c* may reappear or the other bands may disappear until the normal four-banded (reduced) or bandless (oxidized) cytochrome is reformed. In certain conditions, therefore, one part of the cytochrome may be reduced, while the other is oxidized.
- (4) The thoracic muscles of bees spread on a slide and allowed to dry, show according to the spot examined, either all the four bands (*a*, *b*, *c* and *d*), or the bands *a*, *c*, *d*, or *c* and *d*, all the bands occupying their normal positions, or even no bands at all. But the combination *a*, *c*, *d* is most frequently seen. On slightly wetting the muscles and adding a reducing agent, all the four bands of normal cytochrome reappear. The result of this experiment shows that drying does not equally affect the rate of oxidation and reduction of the three components, and that here, contrary to experiment (3), the component *b* is the most rapidly oxidized.

Experiments (3) and (4) show that although under normal conditions *in vivo* the oxidation or reduction of all three components is synchronous, in abnormal conditions, the three compounds reveal a certain degree of independence.

- (5) Finally, Hans Fisher, in collaboration with Schneller and Hilger (1924), has obtained coproporphyrin and Kammerer's porphyrin from yeast. These two distinct porphyrins are probably derived from two of the hæmochromogens found in yeast.

When we consider that the three compounds (*a'*, *b'*, *c'*) forming cytochrome are independent, and probably differ in such of their properties as solubility in reagents and even in their stability, we can readily understand the difficulty of extracting cytochrome in "unmodified" form. It is quite possible that

the paramount conditions for existence of this pigment are found in some properties connected with the physico-chemical structure of the cell. When the latter is modified the component parts begin to display some of their individual properties. When the structure is completely changed, the complex compound undergoes further modification and yields finally as derivatives three hæmochromogens A, B, and C. As to the nature of the three components of cytochrome, Messrs. Anson and Mirsky inform me that they have definitely proved that the components *b'* and *c'* have a hæm- nucleus (iron-pyrrol compound) identical to that of hæmoglobin. They have no certain evidence yet as to the composition of the component *a'*. This shows that while *b'* and *c'* and their derivatives in KOH can be considered as hæmochromogens (hæm+ nitrogen compound), the hæmochromogen-nature of the compound *a'* or A is not yet definitely established.

#### *Cytochrome as a Peroxidase.*

Cytochrome and its derivatives have the properties of a thermostable peroxidase. All the tissues where cytochrome is present and can be seen spectroscopically give a good reaction with benzidine or with guaiacum and  $H_2O_2$ . The tissues of invertebrates, where the concentration of this pigment is very low, give a very weak reaction or none at all. In the same animal, such as *Dytiscus*, the brown muscles, rich in cytochrome, give a strong positive peroxidase reaction, while the white muscles which are very poor in cytochrome give hardly any reaction.\*

#### *Cytochrome and Muscle-hæmoglobin (= myochrome = myoglobin) in Vertebrates.*

The study of organisms containing cytochrome as the only respiratory pigment seen spectroscopically has enabled me to establish beyond doubt the main properties of this pigment and of its derivatives. We shall now be able to pursue, without any risk of confusion, the study of this pigment in animals such as vertebrates, which are provided with hæmoglobin in their blood and tissues. This, on the other hand, will bring us into touch with the very important and much discussed problem of the hæmoglobin of muscles.

It has long been known that the muscles of vertebrates vary greatly in the intensity of their coloration. But it was only during the early investigations on the total amount of blood in animals that it was found that the colour of the

\* The localisation of the peroxidase in relation to the distribution of cytochrome in the cells, together with the study of various inhibitors of this reaction, will be dealt with in a separate paper.

muscles is mainly due to the pigment actually located in muscular tissue.\* The nature of the pigment was, however, much discussed and opinions thereon divided into two groups: some authors, with Kühne (1865) considered it as ordinary hæmoglobin and derived from blood-vessels, others with Kolliker (1860), regarded it as a special pigment actually formed within the muscles. Mörner† described it under the name of *myochrome*, showing that the positions of its two bands differ from those of hæmoglobin by being shifted towards the red side of the spectrum. The middle of the bands in myochrome occupies  $581\cdot5/543\cdot5$   $\mu\mu$ , those of ordinary hæmoglobin  $577\cdot5/540\mu\mu$ .

More recently this pigment was described by Gunther (1921) under the name of *myoglobin* and he mentions also several of its derivatives. From Gunther's description it is obvious that he was dealing with a pigment similar to Mörner's myochrome, which differs very little from ordinary hæmoglobin. He confused it, however, with MacMunn's myohæmatin and, taking for granted that these two pigments are similar, he disagreed with the objections raised against myohæmatin by Hoppe-Seyler and Levy. Without examining the myohæmatin found by MacMunn in insects, however, he assumed that it was identical with his own metamyoglobin. Finally, when he examined the muscles of mammals previously soaked in water, and found there two bands of the real myohæmatin of MacMunn, he failed to recognize them and referred them to some unknown pigments. This almost inextricable confusion of two entirely distinct pigments has crept into the recent literature and MacMunn's myohæmatin seems to have suffered more harm from Gunther's effort to revive it than it ever endured from Hoppe-Seyler's destructive criticisms.

### *Preliminary Experiment.*

Before we proceed to study cytochrome in the tissues of vertebrates, I must describe a preliminary experiment which shows how the absorption spectrum of cytochrome can be observed in spite of the presence of hæmoglobin. A few drops of dilute laked sheep's blood are added to 5 c.c. of an emulsion of yeast, which rapidly reduces  $\text{HbO}_2$  to Hb. The amount of blood added should reach such a concentration as to show with the microspectroscope a very faint band of reduced hæmoglobin. Shaking this mixture with air and rapidly examining it with the microspectroscope, we can observe the appearance of the

\* For the history of early work on this subject the reader is referred to the papers of Kühne, Levy and Gunther.

† Unfortunately Mörner's paper was not accessible to me, and the information mentioned here is derived from the paper by Gunther.

bands of oxyhæmoglobin, while the cytochrome of the yeast, being oxidized, does not show absorption bands. Keeping the mixture under observation we can also witness later the reduction of both pigments: the bands of  $\text{HbO}_2$  rapidly disappear, being replaced by a very faint band of Hb, while the four bands of the reduced cytochrome make their appearance and rapidly reach the maximum of their intensity. This experiment can be repeated many times with the same result.

The contrasting facts that cytochrome shows the absorption bands only in its reduced form, while dilute hæmoglobin shows them in its oxidized form, are of great value in enabling the two pigments to be distinguished even when they are mixed together, provided that the concentration of hæmoglobin is not too strong.

#### *Cytochrome in Frog.*

The muscles and other tissues of a perfused frog are completely devoid of hæmoglobin. Cytochrome is, however, present, its highest concentration being found in the ventricle of the heart and in the testes. It may also be seen in the walls of the intestine and in the leg muscles when the latter are examined in very thick layers, and especially on addition of a reducer ( $\text{Na}_2\text{S}_2\text{O}_4$ ). The concentration of cytochrome in the tissues of this animal is, on the whole, very low, *e.g.*, the heart of a frog actually contains a less quantity of this pigment than the thorax of a bee.

#### *Cytochrome and Muscle Hæmoglobin in Guinea Pig.*

The muscles of this animal, no matter how carefully it is perfused, always show the two bands of  $\text{HbO}_2$  when exposed to air. When, however, their positions are carefully determined, it is found that they do not coincide with the bands of  $\text{HbO}_2$  of the blood of the same animal, *e.g.*, the  $\alpha$ -band of  $\text{HbO}_2$  of blood, corresponding approximately to 5765, while the position of  $\alpha$ -band of  $\text{HbO}_2$  in the muscle is 5800. This observation agrees with previous results obtained by Morner and Günther, inasmuch as the muscular tissue of a perfused animal contains a slightly different hæmoglobin from that of its blood. This difference does not justify, however, the introduction of the names "myochrome" or "myoglobin" for muscle hæmoglobin proposed respectively by Morner and Günther. In fact, the difference between muscle and blood hæmoglobin is no greater than that which is known to exist between the blood hæmoglobin of different animals (*cf.* Anson, Barcroft, Mirsky and Oinuma, 1924). The introduction of such a term as myoglobin would be merely a bad

precedent for the creation of as many names as there are groups of hæmoglobin showing slight differences in the position of their bands.

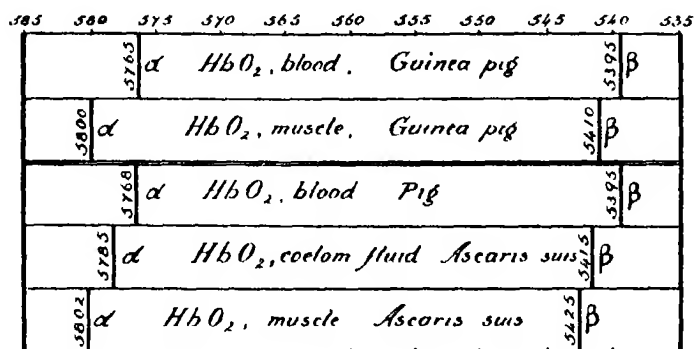


FIG. 5.—Positions of  $\alpha$  and  $\beta$  bands of oxyhaemoglobin in blood and muscles of guinea-pig and *Ascaris suis*.

A slice of the heart or other muscle of a perfused guinea-pig, simply put on a slide uncovered and examined with the microspectroscope, shows the two characteristic bands of muscle hæmoglobin. If now this muscle is covered with another slide, it will soon show, at least in its central area, the HbO<sub>2</sub> becoming gradually reduced, the two bands fading away, while the four bands of reduced cytochrome make their appearance. This change in the spectroscopic aspect of the muscle gradually spreads from the central area towards the periphery, but is completed only when the free space between the two slides surrounding the muscle is filled with glycerine or liquid paraffin. In this experiment we see the repetition of what we have already seen to occur in the mixture of yeast and hæmoglobin. The muscles of a perfused guinea-pig contain thus two pigments: (1) muscle hæmoglobin and (2) cytochrome.

When this muscle is again exposed to air, the Hb becomes oxidized and its two bands reappear, while those of cytochrome disappear. The reduction and the appearance of the bands of cytochrome can be accelerated if Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> is added to the muscle. We can see now that the peculiar bands of a pigment which were observed by Günther in the pulp of muscle extracted with water belong to the remains of the cytochrome. This experiment also shows the mistakes in the experimental method of Kuhne (1865), who sought to prove that the muscle of a turtle, compressed between two slides and brought to tetanic contraction, reduces its hæmoglobin, though the latter becomes re-oxidized when the muscle is re-exposed to air. It is most probable that in this experiment the mere keeping of the muscle between two slides was

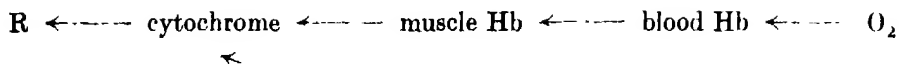
responsible for the reduction of  $\text{HbO}_2$  through the action of the muscle itself, even before it was induced to contraction.\*

*Cytochrome and Muscle Hæmoglobin in Pigeon.*

Perfused pectoral muscles of a pigeon, washed in Ringer solution and examined with the microspectroscope show the presence of a great amount of muscle hæmoglobin, as well as cytochrome. The position of the  $\alpha$ -band of  $\text{HbO}_2$  of muscle is 5805, while that of  $\text{HbO}_2$  of blood 5763. The four bands of cytochrome show approximatively similar positions to those in the muscles of a guinea pig. An interesting character peculiar to the muscles of a pigeon is their great reducing power. A slice of such muscle, even when exposed to the air, always shows the pigments partially reduced, and in order to make the bands of  $\text{HbO}_2$  clearly visible it is necessary to add to the muscle a very dilute KCN solution, which breaks the oxidation system of the tissue. The bands of  $\text{HbO}_2$  also appear on adding a small amount of  $\text{H}_2\text{O}_2$ , which, on being decomposed by a catalase, oxidizes the muscles. The great reducing power of the pectoral muscles of the pigeon explains the main error of MacMunn, who, after having overlooked the presence of hæmoglobin in these muscles, selected them for the extraction of his "modified myohæmatin." It is certain that the product of this extraction was a mixture of derivatives obtained both from cytochrome and muscle hæmoglobin. Levy and Hoppe-Seyler were correct in assuming that the pectoral muscles extracted by MacMunn contained hæmoglobin. They both, however, were wrong in stating that hæmochromogen with its oxy- and carboxy-compounds could not possibly be derived from any other pigment than hæmoglobin. In fact, as we have seen previously, cytochrome derived from organisms which are completely devoid of hæmoglobin (insects, yeast, etc.) yields characteristic hæmochromogens giving  $\text{O}_2$  and CO compounds.

The above observations clearly show that the muscle of a living mammal, bird, and probably reptile, contains the following respiratory pigments:— (1) Cytochrome and (2) muscle-hæmoglobin (= myochrome = myoglobin) in the muscular tissue itself and (3) blood hæmoglobin in the vessels. The transmission of  $\text{O}_2$  through this system can be represented schematically as follows:—

\* It must be mentioned that cytochrome can be observed in the muscles of a freshly-killed rabbit, prepared without perfusion, but only by careful washing of the muscles in warm Ringer's solution. It can be easily seen also in the nerve-tissue, such as the properly washed brain of a sheep.



R being the oxidized substances in the cells

### *Cytochrome and Hæmoglobin in Invertebrates.*

The co-existence of cytochrome and hæmoglobin may occur also in invertebrates, a great number of which, as shown by Ray Lankester, contain hæmoglobin in the blood, body cavity or tissues. It may be mentioned here that even the existence of two kinds of hæmoglobin in the same animal is not restricted to vertebrates, as I have found it in a Nematode worm, *Ascaris suus*, an intestinal parasite of pigs. This worm is reddish in colour and its perivisceral or body fluid contains  $HbO_2$  with the absorption bands lying respectively at:  $\alpha$ -5785,  $\beta$ -5115. The muscles or the integument of this worm, carefully washed clear of the body fluid, remain pink and show the absorption bands of  $HbO_2$  nearer the red end of the spectrum ( $\alpha$ -5802  $\beta$ -5125). The position of the bands in body fluid of *Ascaris* is approximately intermediate between that of the pig's blood and the  $HbO_2$  in the integument of the worm (fig 5).

Among the invertebrates hæmoglobin is distributed in a rather haphazard way, being present either in all representatives of a family (in Annelids) or being confined to one or more genera or species, while the remaining forms of the same family are completely or almost completely devoid of it (Insects, Molluscs). On the other hand, the mere presence of a hæmoglobin in an organism does not necessarily imply the existence of a deep physiological difference between this organism and another which is devoid of this pigment. In fact, taking into consideration the general distribution of cytochrome and of its components, we may say that most, if not all, organisms already possess the principal constituents from which hæmoglobin may arise. In other words, a very great number of organisms are potential carriers of hæmoglobin.

### *Hæmochromogen and Cytochrome in Higher Plants.*

The material for this study consisted of non-coloured portions of various plants: bulbs of eschalots, garlic and leek; potatoes, grains of wheat, barley and oats, various beans and stamens of young flowers of narcissus, hyacinths, and crocuses.

A section of 3-6 mm. thick cut from the base of an eschalot bulb examined with the microspectroscope shows three absorption bands, the centre of the



strongest lying at  $556 \mu\mu$ , a second band, although faint, is always visible at  $524 \mu\mu$ , and in few cases a very faint shading can be seen at  $603 \mu\mu$ . The pigment responsible for this absorption band is a hæmochromogen-like complex, which we shall call a modified cytochrome. When the same section of eschalot is treated with a reducer  $\text{Na}_2\text{S}_2\text{O}_4$  the four bands (*a*, *b*, *c*, *d*) of cytochrome slowly appear, the bands are faint and their positions resembles those of bakers' yeast, with the exception that the bands *b* and *c* seem to be a little nearer one another and the space between them is slightly

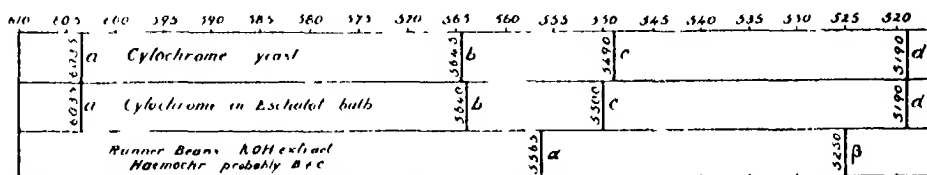


FIG. 6.—Positions of the bands of cytochrome and its derivative in yeast, eschalot, and beans.

shaded. In similar way the bands of cytochrome can be observed in all bulbs of other monocotyledons, in beans, and pollen of plants. The KOH extract of plant tissues give hæmochromogens resembling those of yeast, combining with both  $\text{O}_2$  and  $\text{CO}$ . It is obvious that the organic iron compounds which yield pyrrol as derivative, obtained by Gola (1915–1919) from various plants, derive from the hæmochromogen or cytochrome complex, which as we have seen are widely distributed in plants.

I cannot say definitely yet what may be the relationship between the hæmochromogen-like complex and the cytochrome in plants. It is very probable that the former, which is invariably seen in plants previous to treatment with a reducer, already contains the three components of cytochrome, and differs from the latter in the constitution of its component *b'*. This component having here (*b''*) its  $\alpha$ -absorption band nearer to that of the component *c'*, mixes probably with it, both forming a strong band with the centre at  $556 \mu\mu$ . In this case  $\text{Na}_2\text{S}_2\text{O}_4$  by bringing out the bands of reduced cytochrome would not act as a reducer but as a substance changing the properties of the cells, inducing thus the hæmochromogen *b''* to modify and to give rise to the corresponding components (*b'*) of cytochrome. This supposition is corroborated by the following observations :

- (1) The appearance of the absorption bands of cytochrome in plants is accompanied by the gradual disappearance of the bands of the hæmochromogen-like complex.

- (2) This phenomenon takes place not only when the reducer is added to the tissue, but also when tissues are treated with a strong solution of NaCl, or when they are simply dried.\*

#### *Cytochrome in Bacteria.*

A thick emulsion of aerobic bacteria, such as *Bacillus subtilis*, examined with the microspectroscope, shows the four characteristic bands of cytochrome. When this emulsion is shaken with air the cytochrome becomes oxidized and the bands disappear. Anaerobic bacteria, on the other hand, such as *B. sporogenes* are devoid of cytochrome, showing once more that cytochrome is a respiratory catalyst connected with the aerobic mode of life.

#### *Summary.*

1. Cytochrome is an intracellular respiratory catalyst common to animals, bacteria, yeast and higher plants.
2. In a reduced state it shows a characteristic absorption spectrum with four bands, the position of which in all organisms are approximatively the same: *a*-6046, *b*-5665, *c*-5502, *d*-5210 Å (bees). Band *d* is composed of three secondary bands, *x*, *y* and *z*.
3. In the oxidized form there are no clear absorption bands but only faint shading extending 520-540/550-570.
4. The highest concentration of the pigment is found in the thoracic wing muscles of flying insects, striated muscles of mammals and birds, and bakers' yeast.
5. Cytochrome is easily oxidized with air and reduced by the normal activity of cells or by a chemical reducer.
6. KCN in concentration of  $n/10,000$  or sodium pyrophosphate stops the oxidation of cytochrome, but these substances do not prevent the cell reducing the already oxidized form of the pigment.
7. Ethyl urethane, alcohol and formaldehyde do not interfere with the oxidation of cytochrome, but they inhibit the reducing power of the cell, and the cytochrome thus remains oxidized.
8. Under natural conditions in the living animal cytochrome is in the oxidized or in only partially reduced form.
9. The condition of cytochrome as seen spectroscopically in the living

\* Cytochrome and its derivatives in plants and bacteria will be dealt with in more detail in a separate paper.

organism denotes only the state of equilibrium between the rate of its oxidation and reduction at that particular time.

10. The behaviour of cytochrome in living organisms is dealt with in detail.

11. Evidence is brought forward that cytochrome consists of three hæmochromogen compounds ( $a'$ ,  $b'$ ,  $c'$ ) two of which ( $b'$  and  $c'$ ) have a hæm. nucleus (iron-pyrrol compound) similar to that of hæmoglobin.

12. Cytochrome yields as derivatives three hæmochromogens which give  $O_2$  and CO compounds.

13. Cytochrome and its derivatives are responsible, at least partly, for the peroxidase reactions in organisms.

14. Cytochrome is distinct from muscle-hæmoglobin (= myochrome = myoglobin) and both pigments can be easily seen in the same muscle of a bird or a mammal.

15. Muscle  $HbO_2$  differs from blood  $HbO_2$  only in having the absorption bands slightly shifted towards the red end of the spectrum.

16. The non-coloured portions of plants show the existence of a hæmochromogen-like complex (modified cytochrome) ( $a'$ ,  $b''$ ,  $c'$ ), as well as of cytochrome, both yielding in strong KOH characteristic hæmochromogens which give  $O_2$ - and CO-compounds.

17. Cytochrome exists in aërobic bacteria and can be oxidized and reduced as in other organisms.

The expenses of this research were defrayed by the Medical Research Council. I wish to thank Mr. J. Barcroft for his constant interest in the progress of this study. My thanks are due to Messrs M. L. Anson and A. E. Mirsky for the great help they have given me in connection with this investigation. To Dr. I. M. Puri I owe friendly assistance in numerous dissections and the perfusion experiments.

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### CROONIAN LECTURE.—*Animal Posture.*

By RUDOLF MAGNUS, Professor of Pharmacology, University of Utrecht.

(Lecture delivered June 11,—MS received June 16, 1925)

Every movement starts from and ends in some posture, so that I think a discussion of "Animal Posture" falls well within the scope of the intention of Dr. William Croone, when he founded these annual Lectures to promote the study of "Muscular Motion." Before beginning I wish to emphasise how greatly I appreciate the honour of delivering before you this Lecture, and how I especially enjoy the pleasure of doing so with Sir Charles Sherrington in the Chair, who long ago took the trouble to introduce me to his beautiful methods of investigating the central nervous system, and to allow me an insight into his fruitful views on the function of nervous centres.

As it is impossible to consider the whole problem of posture in one short lecture, I propose to speak to you to-day on four partial problems, which are closely connected with each other, and which provided the starting points for investigations which have been carried out in my laboratory at Utrecht, with the aid of a great number of able collaborators. These partial problems are:—

1. *Reflex standing.*—In order to carry the weight of the body against the action of gravity, it is necessary that a certain set of muscles, the "standing muscles," should have by reflex action a certain degree of enduring tone, to prevent the body from falling down on the ground.

2. *Normal distribution of tone.*—In the living animal not only do these standing

muscles possess tone, but also the other muscles of the body, especially their antagonists, *i.e.*, the flexors. Between these two sets of muscles a certain balance of tone exists, so that neither set of muscles gets too much or too little tone.

3. *Attitude*.—The position of the different parts of the body must harmonise with each other ; if one part of the body be displaced, the other parts also change in posture, so that different well-adapted attitudes, evoked by the first displacement, will result.

4. *Righting function*.—If by its own active movements or by some outside force the body of an animal is brought out of the normal resting posture, then a series of reflexes are evoked, by which the normal position is reached again.

The main *centres* for all these four functions are situated in close neighbourhood subcortically in the brain-stem. Their function is to compound the activity of the whole body musculature to what we call "*posture*." The lower centres for the muscles of the different parts of the body are arranged segmentally in the spinal cord ; the higher centres in the brain-stem put them into combined action, and in this way govern the posture of the animal as a whole. We have here a very good example of what Sherrington has called the "integrative action of the nervous system." And integration is especially necessary in the case of posture, because nervous excitations arising from very different sense organs are flowing towards the postural centres in the brain-stem, and must be combined so that a harmonising effect will result.

Nervous impulses, which can influence posture, arise : (1) From the labyrinth, a double sense-organ : the otoliths reacting to changes in position, the ampullæ of the semicircular canals to accelerations ; (2) from the proprioceptive sense-organs in muscles, joints and tendons ; (3) from exteroceptive nerve endings of the body surface, chiefly from the pressure sense-organs, which are stimulated, if the body touches the ground ; (4) from teleceptors, reacting to distance stimuli, such as the eye, the ear, the nose. In fact a very finely elaborated central apparatus is needed to combine and distribute all these afferent impulses, depending on and adapted to the always changing circumstances of environment.

After these introductory remarks we can turn to our subject itself and first discuss "*reflex standing*." It is not necessary to go into any details about that problem before this Society, as we owe to your President the fundamental investigations which form the solid basis for all work on this question. So a few remarks will be sufficient.

If we try to put the body of a dead animal upon its feet, the carcass immediately falls down to the ground, because the relaxed muscles cannot carry the weight of the body against the action of gravity. The same happens with a living animal after total extirpation of the brain, so that only the spinal cord is in functional connection with the muscles. Such a "spinal animal" can, of course, perform movements with a high degree of perfection. After pinching one foot, we see the limb retracted away from the stimulus; a spinal preparation, suspended in the air, will perform with its hind legs regular alternating movements resembling closely the running movements of the intact animal; by rubbing the skin gently a scratch reflex of apparently normal shape and frequency is evoked; distension of the lowest parts of the bowel causes all the complicated movements which accompany normal defecation. The centres of the spinal cord can indeed cause and regulate very complicated combinations of movements, but they are unable to give to the muscles that steady and enduring tone which is necessary for simple standing.

It has been shown by Sherrington that this state of affairs is fundamentally changed, if the spinal cord be in functional connection with the hind part of the bulb. By cutting across the brain-stem somewhere between the posterior half of the medulla oblongata and the foremost part of the mid-brain, the "decerebrate rigidity" arises, in which the standing muscles acquire an abnormally high degree of tone. These anti-gravity muscles are the extensors of the limbs, the extensors of the back, the elevators of neck and tail, and the closing muscles of the jaw. The antagonists of these muscles, *i.e.*, the flexors, have, on the contrary, either no tone at all or an abnormally low degree of tone. The consequence is, that the decerebrate preparation will *stand*, if it has been put upon its feet, but stands in an *abnormal posture*, with exaggerated extension of limbs, neck and tail. The distribution of tone is an abnormal one. The stimuli inducing the enduring tone of the standing muscles in decerebrate rigidity arise from different sources, the proprioceptive sense-organs in the contracted muscles themselves playing the most prominent rôle.

In order to get a *normal distribution of tone* the presence of the foremost part of the mid-brain is necessary. By cutting the brain-stem somewhere in front of the mid-brain or by extirpation of the fore-brain, so that a "mid-brain animal" or a "thalamus animal" results, one gets preparations in which the standing muscles still have tone, but not an exaggerated one, as after decerebration, but a normal one as in intact animals. On the other hand, the flexors are no longer toneless, but have as much tone as a normal animal usually has.

A thalamus animal therefore will stand in a "normal" posture and not in the hyperextended posture of the decerebrated preparation.

It has been found in careful experiments by Dr. Rademaker in Utrecht, that the presence of the "red nucleus" is essential for the change from decerebrate rigidity to normal distribution of tone, and that the rubro-spinal tract carries the impulses down to the spinal cord, by which this difference is called forth. In the thalamus animal the extensors of the limbs just have sufficient tension to balance the weight of the body against gravity, so that every force tending to raise or to lower the body can easily move it in one or the other direction.

The motor centres of the cortex cerebri have a similar but by no means so strong an influence, which is exerted by way of the pyramidal tract. They also tend to diminish decerebrate rigidity, but are much less important for this function than the system of the red nucleus.

I have discussed the problem of standing hitherto in a more general way, speaking of the body as a whole. Still more interesting is the investigation of the rôle which the different parts of the body, each for itself, play in the joint function of standing. The relation in position of all the parts of the body to each other forms the basis of *attitude*. In normal animals and man the positions of the different segments and parts are always in harmony, and it is easy even for a layman to detect what is a "normal," a "pathological" or a "caricature" posture. As soon as the position of one part changes, the other parts of the body also acquire a new position; the result is a new attitude, which in a normal individual is again harmonious.

The most fundamental attitudinal reflexes can best be studied in the decerebrate preparation. We put such an animal on its feet and pinch one hind foot; the stimulated limb is retracted from the ground and the hind part of the body is no longer carried by two, but only by one limb. Nevertheless, it does not fall, because together with the homolateral flexion reflex a crossed extension reflex of the opposite limb is evoked, by which the limb is strongly stretched and is able to carry the weight quite alone. This is what one could call a "segmental" attitudinal reflex, in which only one segment of the body is concerned. Several segmental reflexes of that kind have been studied. But more important are the general attitudinal reflexes, by which the whole body is influenced.

It is noteworthy that these reflexes are most easily evoked from the foremost part of the body, from the head, in which the teleceptive sense organs

are situated, so that distance stimuli influencing the position of the head can in this way also impress different attitudes upon the whole body. One can, in fact, in the decerebrate animal, by simply changing the position of the head, give to the body a great number of attitudes, resembling closely the normal harmonious attitudes of the intact animal. The analysis of these reactions, which I had the opportunity of effecting in co-operation with Dr. A. de Kleyn, showed that they are the result of the combined action of two sets of reflexes, *tonic labyrinthine and tonic neck reflexes acting on the body muscles*. That is what one would expect, as in changing the position of the head one performs two separate things: (1) changing the position of the head *in space*, and therefore stimulating the otolithic apparatus; (2) changing the position of the head *in relation to the body*, therefore flexing or twisting the neck and stimulating the proprioceptors of the deep structures of the neck.

In order to study the tonic labyrinthine reflexes, one has to exclude the neck reflexes, either by cutting the first three posterior sensory roots or by bandaging head, neck and thorax, so that no neck movements are possible. If one brings such an animal into different positions in space, only one position of the head is found in which the tone of the standing muscles is maximal—supine position of the head, with the snout a little above the horizontal plane. There is another position of the head, differing from the first one by 180°, in which the tone of the standing muscles reaches a relative minimum, so that some tone is still present, but less than with the head in any other position. In all other positions of the head in space the tone of the standing muscles assumes values between those extremes. Under the influence of the tonic labyrinthine reflexes the tone of all the standing muscles always changes in the same direction. The flexors are influenced in the opposite sense. With maximal tone of the extensors the flexors are (in the decerebrate preparation) relaxed; with minimal tone of the extensors they may acquire some tension.

In order to study the tonic neck reflexes alone, one has to exclude labyrinthine reflexes by labyrinth extirpation. Under these conditions flexing, bending, twisting of the neck evokes tonic reflexes, in which usually one-half of the body reacts in an opposite way to the other, *e.g.*, the fore-limbs being extended, the hind-limbs relaxed, and *vice versa*. But there are also neck reflexes, with which all four limbs react in the same sense.

If both sets of reflexes are present, the result is a very complicated one, because the same movement of the head in relation to the body will, with different positions of the body in space, give rise to different labyrinthine reflexes; therefore one has to study the effects of all different movements of



the head in all different positions of the body. It has been possible to refer all attitudinal reactions, observed in very numerous experiments, to the co-operation of labyrinthine and neck reflexes. The general rule is, that every group of muscles reacts to the algebraic sum of stimuli arising from the labyrinth and neck receptors, so that if the extensors of one fore-limb, for example, get increased tone from both the labyrinths and the neck, the limb will be strongly stretched, whereas if it gets increased tone from the labyrinths and decreased tone from the neck, it may remain unchanged altogether.

The centres for these attitudinal reflexes are the most caudally situated ones of all postural centres: those for the tonic neck reflexes in the two highest cervical segments, those for the tonic labyrinthine reflexes in the hind part of the bulb, behind the plane of entrance of the VIII. cranial nerves. These reflexes are called tonic, because they last as long as the head keeps a certain position; and that not only for minutes and hours, but for days, months and even years. After unilateral labyrinth extirpation in the rabbit turning of the neck results, which evokes the normal tonic neck reflex, one fore-limb being extended, the other flexed. If the other labyrinth is not extirpated in a second operation, in order to put the head right again, the turning of the head will last as long as the animal lives, and the tonic neck reflex at least several months. We are accustomed to believe that muscular action is liable to fatigue, and this, of course, is true for movements, and especially for movements performed against resistance. But muscular action concerned in keeping some part of the body in constant and unchanging position gives rise to much less fatigue, and the attitudinal tonic reflexes evoked from the head appear to be practically indefatigable.

Now the question arises, what use the normal, not decerebrated animal makes of these reflexes. A normal rabbit sits usually in a squatting posture with head down, fore-limbs flexed and back curved. By taking the head and flexing it dorsally, so that the snout is raised, a combined labyrinthine and neck reflex is evoked, by which both fore-limbs are strongly stretched, the fore part of the body raised and the back extended. It is not even necessary to initiate this reaction by a passive movement of the head. The latter can be made to move actively by means of the distance receptors. If a cat is sitting in a cage and a piece of meat is held low down near the ground, the animal fixes the food with its eyes, bends the head in the ventral direction, evokes labyrinthine and neck reflexes, which tend to flex the fore-limbs, and the whole body of the animal is in this way directed towards the food. By moving the meat upwards, one makes the animal follow the food with its eyes, flex the neck

dorsally and give rise to labyrinthine and neck reflexes, by which the fore-limbs are strongly extended, the fore part of the body raised; whereas in the hind limbs, neck and labyrinthine reflexes just compensate each other, so that no change in their posture occurs. The result is a somewhat "monumental" attitude, by which the body of the animal is again directed towards the meat and at the same time brought into a posture which enables it to jump and to catch its prey.

Suppose a cat is standing in the middle of the room, and a mouse is running on its right side along the wall. The optic and acoustic stimuli act on the telereceptors of the cat's head, and make it turn the heavy head to the right. By this the centre of gravity of the fore part of the body is displaced to the right. At the same time tonic neck reflexes are evoked, by which the vertebral column is curved and the right fore-limb strongly extended, so that it carries the weight of the body alone and prevents it from falling. The left fore-limb has nothing to carry, and in harmony therewith this limb relaxes under the influence of the tonic neck reflex. At the same time the distribution of excitability in the motor centres of the spinal cord is rearranged by the turning of the neck, so that, if for some reason running movements begin, the limb which has no static function will always make the first step. In this way the moving mouse impresses on the cat through the mediation of tonic neck reflexes an attitude, by which the cat is focussed towards the mouse and made ready for movement. The only thing the cat has to do is to decide: to jump or not to jump; all other things have been prepared beforehand reflexly under the influence of the mouse, which will be the object of the resulting jump.

These examples may give an impression of the different ways in which the tonic labyrinthine and neck reflexes are used during the normal life of intact animals. They can easily be extended by watching various kinds of animals. Only in the monkey they cannot easily be detected, because with the higher development of the fore-brain and the greater complication of movements these lower postural reflexes are partly suppressed. It is necessary to decerebrate or narcotise a monkey in order to show that he has the same attitudinal reflexes, obeying the same laws, as are shown by other animals.

Also, in man during ordinary life the attitudinal reflexes cannot easily be detected. In the infant child several of them are present. In adults instantaneous photographs show sometimes postures in agreement with the laws of attitudinal activity of the brain-stem centres. I owe to Dr. Wolf the acquaintance with fast cinema photos of golf players, showing that at the top of the swing the shoulders are turned by 180° in relation to the head, which is kept

fixed in space as firmly as possible. In this way a tendency is evoked to extend the left arm and to twist the body to the left—movements which appear to be performed, indeed, by the subsequent photographs, and which seem to be facilitated and strengthened by the preliminary starting posture of the head and body. It is this fixing of the head in the line of sight of the ball which is insisted upon as essential to the performance of a correct shot.

Many masterpieces of painting or sculpture representing human beings are consistent with the laws of attitudinal reflexes. But under pathological conditions, especially if the function of parts of the cerebrum is disturbed, they are released, and are now used frequently by neurologists for diagnostic purposes. Thus, in some cases of chronic hydrocephalus, turning of the head causes the arm (or leg) towards which the face is turned, to be extended as long as the head keeps this position, but to relax as soon as the head turns to the other side. There are not so many cases in which tonic labyrinthine reflexes have been demonstrated with certainty; one of these was a patient with amaurotic idiocy, who exhibited in one position of the head in space flaccidity, in another strong tonic extension of both arms and legs, neck reflexes being excluded by firmly bandaging head, neck and thorax, so that no neck movements were possible.

Every change of attitude, with its different positions of all the parts of the body, changes the reflex excitability of these parts, and in some cases changes also the sense of the reflex evoked, excitations being converted into inhibitions, reflex extensions into flexions, and so on. One and the same stimulus applied to one and the same place on the body may give rise to very different reactions, in consequence of different attitudes which have been imposed to the body before the stimulus is applied. The laws governing such changes of reaction have been greatly elucidated, but the results are somewhat complicated, so that they cannot be summarized in a short lecture. Only one case may be mentioned. It sometimes happens that in consequence of shock or inhibition a certain movement of the head will not evoke the tonic neck reflex we should expect. In this case an indifferent stimulus, which under ordinary conditions would never evoke postural reactions, can induce the tonic reflex which the movement of the head was not able to provoke.

It is interesting that the same phenomenon has been found in human patients by two independent observers—Dr. Simons of Berlin, and Dr. Walshe of London. After shot-wounds of the brain, with consequent unilateral paralysis, strong movements of the hand on the normal non-paralysed side evoke involuntary associated movements of the paralysed arm and leg.

Turning of the head in many of these patients fails to give rise to direct tonic neck reflexes affecting the paralysed limbs, but influences the direction of the associated movements. If the face is turned towards the paralysed side the associated movement will be extension, whereas flexion results if the face is turned towards the sound side. In this way the position of the head determines the sense of movements caused by indifferent stimuli.

The foregoing examples are sufficient to give an impression of the importance of attitudinal reflexes in animals and human beings under normal and pathological conditions.

The decerebrate animal has no *righting function*. Put on its feet it stands, but if it receives a blow, it falls on its side and is unable to return to the normal standing position. The mid-brain animal behaves in a very different manner. Not only is the distribution of tone a normal one, but also the righting function is fully developed, and the animal is able, from all abnormal positions, to come back reflexly into the normal position. The reflexes which co-operate in attaining this result are the "righting reflexes." They can best be studied in the mid-brain animal, or thalamus animal, in which the fore-brain has been removed, so that no voluntary corrections of abnormal sensations are possible. We have to deal with a pure reflex machinery of a very high degree of perfection.

The first group to be described are the *labyrinthine righting reflexes*, evoked from the otolithic apparatus and tending to bring the head into and to keep it in the normal position. In order to give an isolated demonstration, one has to hold the body of a thalamus animal (*e.g.*, a rabbit) freely in the air, so that it does not touch the ground. Suppose the animal is held by the pelvis, one can turn the latter from one lateral position to the other and still the head is kept in normal position. The same holds good if the animal is hanging with head down, or if the pelvis is turned into the supine position, and so on. Whatever situation one gives to the hind part of the body, the head is kept, as by a magic force, in its normal position in space. This magic force is provided by the stimuli arising from the otolithic apparatus. By labyrinth extirpation, or by centrifuging away the otolithic membranes from their maculae, these righting reflexes are abolished, the head is now absolutely disorientated in the air, and will remain in any position (lateral, supine position, and so on) according to the position of the hind part of the body.

Suppose I hold the pelvis of a delabyrinthized thalamus animal in the lateral position in the air, then the head will remain also in the lateral position. The

body is now placed (always in lateral position) on the floor. At the moment the body touches the ground, one sees the head righted into the normal position. This reaction is evoked by the asymmetric stimulation of the exteroceptive pressure sense organs of the body surface, as is proved by the fact that the head immediately goes back into the lateral position if a weighted board is put on the uppermost side of the body and the asymmetric stimulation by the ground thus compensated. After the board has been removed, the head is righted again; if the animal is taken away from the floor and held freely in the air, the head falls back into the lateral position. In this way contact with the ground rights the head (*body righting reflexes acting on the head*).

Labyrinthine and exteroceptive stimuli co-operate in bringing the head into the normal position relative to space and environment. If the head has been righted by both or one of these influences, whereas the body remains in an abnormal (*e.g.* lateral) position, then the neck is twisted. This gives rise to excitation of the proprioceptive sense organs of the muscles (joints, tendons) in the neck, so that "*neck righting reflexes*" are evoked, which first make the thorax follow the head and right it into the normal position. Now the lumbar region of the trunk will be twisted, a new reflex caused, and finally the whole body will stand in normal position, following the head.

Our own experience and observations on animals show us, that these neck righting reflexes cannot be the only ones which guarantee the normal position of the body. The latter can stand and can right itself, even when the head is *not* kept in the normal position. So there must be some reflexes capable of righting the body if the head has not been righted beforehand. These reflexes exist and can easily be demonstrated. Hold an animal freely in the air and fix the head firmly in the lateral position. Then without resistance the body may easily be kept in lateral position. If the body now be put on the table, it is righted as soon as it touches the surface, in spite of the head being kept in lateral situation, and in spite of the neck righting reflexes tending to hold the body in the lateral position. The active stimulus is again the asymmetric pressure on the exteroceptors of the body surface, as can easily be proved by the use of a weighted board (*body righting reflexes acting on the body*).

In the case of this very important function everything is doubly secured. The head is righted by labyrinthine and exteroceptive reflexes; the body by proprioceptive and exteroceptive reflexes; exteroceptive stimuli control the normal position of head and body; so that, if one of these apparatuses becomes paralysed, others will still be in function.

These four groups of righting reflexes are the only ones which can be demon-

strated in the thalamus animal. Normal rabbits and guinea-pigs have no other righting reflexes. But the higher mammals such as cats, dogs and monkeys are, with intact cerebrum, in the possession of a fifth set of reflexes: the *optical righting reflexes*. To demonstrate them, one has to hold a delabyrinthized dog or monkey freely in the air; if the attention of the animal is attracted by something in its environment, and it therefore fixes the latter with its eyes, the head is immediately brought to the normal position and kept so as long as the optical attention is focussed on the subject. So a telereceptor has gained influence upon the righting apparatus. This is the only righting reflex having its centres not in the brain-stem but higher up in the cortex cerebri.

The multiplicity of reflexes, causing and maintaining the erect position, makes it intelligible how labyrinthless deaf-mutes can stand and walk without apparent disturbance. Only if brought under water, where the optical impressions cannot be used and no body righting reflexes can be evoked, they are completely disorientated and will be drowned if they are not helped out of the water.

It seems to be of the greatest importance, that the whole central apparatus for the righting function (with the only exception of that for the optical righting reflexes) is placed subcortically in the brain-stem and by this means withdrawn from all voluntary action. The cortex cerebri evokes during ordinary life a succession of phasic movements, which tend over and over again to *disturb* the normal resting posture. The brain-stem centres will in the meantime *restore* the disturbance and bring the body back into the normal posture, so that the next cortical impulse will find the body prepared to start again. It is also an essential condition for the right interpretation of all *sensory* impressions reaching the cortex, that the body be always brought into the normal position by a purely automatic subcortical arrangement, which controls the spacial relation of the body to its environment. In his last anniversary address your President called attention to the importance this arrangement possesses from a psychological point of view.

The knowledge of the righting reflexes permits a better understanding of many pathological and pharmacological conditions. A good example is presented by the asphyxial convulsions. Asphyxia stimulates motor centres in the spinal cord and the bulb. The effect of excitation of these centres varies, however, according to the state of excitability of the righting centres. If the latter are in good functional condition, then the animal will run and jump; if they are semi-paralysed, the animal will roll over, turning over its back or its belly; if they are completely paralysed, it will lie on its side and show tonic

and clonic convulsive fits. We have here the pharmacological parallel to the above-mentioned fact, that postural activities can modify the reactions of the body to subsequent indifferent stimuli.

Mr. Versteegh of Utrecht has studied the disturbance of motor co-ordination following the ingestion of alcohol in rabbits. At the time the first signs of intoxication appear, the righting reflexes acting on the head are normal, and also the body righting reflexes acting on the body. So the head is kept in normal position and the body is able to right itself. But the neck righting reflexes are paralysed, so that, if the head turns in the one or the other direction, the body does not follow, but goes its own way. The result is the characteristic unsteady gait of the intoxicated.

The time available does not allow me to go into details about the working of another extremely well-adjusted central apparatus which governs *the positions of the eyes*.

The resting position of the eyes is not a fixed one, but changes according to the different positions of the head. Therefore a fine regulation by means of the eye muscles becomes necessary, which have to be controlled by sensory impulses. In man and in animals with frontally arranged eyes, the two visual fields overlap, and one eye can therefore control the position of the other by means of optical impulses. This is impossible in animals with lateral eyes, whose visual fields are separate or overlap only slightly. Here a special non-optical reflex arrangement has been developed, which ensures that in the different positions of the head the optical world is not displaced in an irregular way, but keeps a certain relation to the animal, and that the right and left halves of the visual world do not diverge.

This is accomplished, as has been shown by de Kleyn, by combined action of otolithic and neck reflexes, influencing the eye muscles. If a rabbit sits in the normal position, and then by movements not exceeding the normal range brings its head into another position, then the eyes move in the orbit so that the movement of the head is entirely compensated, and the eyes keep their position relative to space. So, in spite of head movements, the visual world does not move and the two halves of the visual environment of the animal do not diverge. This reaction is initiated by another reflex. If the animal brings the head into a new *position*, it makes a *movement*, and, in doing this, stimulates the ampullæ of the semicircular canals, which gives rise to short-lasting motor reflexes acting on the eye muscles. If the animal is in the normal position the movements of the eye-balls, evoked by these reflexes, are always carried

out in such a direction that the *bulbus oculi* is brought into just that position in which it will then be fixed by the compensatory static reflexes which have just been described. The canals begin, the otoliths and neck receptors complete and steady the reaction. a very finely adapted mechanism indeed. The same connection between motor and static activities has been found also in many other postural reactions, as recently in the myotatic reflexes of Liddell and Sherrington.

In this case also as in the case of the righting reflexes an unconscious subcortical mechanism brings the eyes into the right position, so that the optical impressions to be transmitted to the cortical centres carry with themselves the correct spacial basis, prepared for them in advance and—in the case of the rabbit—almost independent of optical stimuli.

The centres regulating the postural functions described are arranged in the brain-stem in three functional groups: (1) the centres of the attitudinal reflexes behind the plane of entrance of the VIII nerves; (2) the centres of the eye-reactions between the entrance of the sensory nerves concerned and the eye-muscle nuclei; (3) the centres of the righting reflexes (with the sole exception of the optical righting centres in the cortex) in the mid-brain. Of the latter, the neck righting centres extend as far down as to the pons and upper part of the bulb, whereas the other righting centres lie at the level of the red nucleus. As Rademaker has shown, the nucleus ruber is indeed the centre for the labyrinth righting reflexes and for the body righting reflexes acting on the body, but not for the body righting reflexes acting on the head. The efferent path of the two first named reflexes is the rubro-spinal bundle, the afferent tracts have still to be found. Thus a first beginning has been made towards translating the physiological facts into anatomical language, and we hope that still further progress will be possible in this direction.

With the exception of the optical righting reflexes, all the reactions described in this lecture have been found to be present in all species of mammals investigated (guinea-pig, rabbit, cat, dog, monkey) and many of them in other vertebrates too. But the use which the different sorts of animals make of these reflexes is very different, and the relative importance of one or the other group of postural reflexes for various species also varies widely. This seems self-evident, if one compares, *e.g.* the simple shape and limited movements of a guinea-pig with the very complicated movements of such an elegant springing and climbing animal as the monkey. Generally speaking, the relative importance of labyrinthine reflexes decreases with increasing develop-



ment, so that guinea-pigs and rabbits have very strong, monkeys and men much less preponderant labyrinthine reactions, while in the monkey the reactions evoked by means of the eyes and by touch and pressure to the exteroceptive nerves play a very prominent rôle.

The short review which I have been able to give you of the present status of some parts of postural physiology shows that the action of the bulbar and mid-brain centres can now be understood in their principal lines, and that the points are known at which still further investigation of detail is wanted. But this picture would be faulty, if it gave you the impression that the whole problem of animal posture had been solved. That is by no means the case. Besides the medulla oblongata and the mid-brain there are other parts of the brain, which have probably also an influence on body posture. Their physiology is unfortunately more or less unexplored. In the first place the *striatum* and *pallidum* may be mentioned in this connection. Neurologists are at the present time greatly interested in these parts of the brain-stem, because in several diseases, such as Parkinson's and Wilson's disease and in certain forms of encephalitis, severe lesions are usually found post-mortem in the basal ganglia. In these diseases postural disturbances play a prominent rôle. Consequently in neurological literature one encounters a general belief in the postural functions of these centres. Unfortunately in most cases also other parts of the brain are found to be involved. Besides that, neither extirpation nor stimulation of the basal ganglia has given any evidence of postural influences arising in the striatum and pallidum. Our knowledge therefore of the rôle which these centres may perhaps play in the maintenance of posture is at most very restricted.

Still worse perhaps is the situation with regard to another very important part of the brain, the *cerebellum*. Experiments have proved that all postural reflexes discussed in this lecture are present and perfectly undisturbed after total extirpation of the cerebellum. Therefore their centres as well as their afferent and efferent tracts are extracerebellar. Through the brilliant investigations of Luciani and others we know that loss of the cerebellum is followed by severe motor and postural disturbances. These symptoms, however, cannot depend on the cerebellum, which has been removed, but are evoked by the rest of the central nervous system, which has been spared. Unfortunately we know not a single function or reflex positively connected with the cerebellum, in such way that it is absent after cerebellar extirpation, and present after ablation of other parts of the brain, as long as the cere-

bellum remains uninjured. Our evidence of the postural activity of the cerebellum is purely negative. The great advantage during the investigation of the postural functions of bulb and mid-brain was, that we could there deal with positive reflexes (righting and attitudinal) which are present as long as the hind part of the brain-stem is intact. I am convinced that as soon as we succeed in finding positive reflexes connected with the cerebellum, it will be possible to elucidate the mystery of this undoubtedly very important part of the Central Nervous System. Only then can the question of the importance of the different *cortical* centres for posture be raised.

In the study of posture therefore it is the same as in other parts of science. Every step of progress makes it possible to formulate new questions and to delimit anew the bounds of the unknown. In proceeding, one reaches summits which do but open new prospects over vast fields yet to be explored.

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*The Photometric Matching Field.—II. The Effect of Peripheral Stimulation of the Retina on the Contrast Sensibility of the Fovea.*

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*Abstract.*

A previous paper showed that peripheral stimulation of the retina with white light may cause a reduction in the limen of contrast perception at the fovea. The present paper extends the investigation to monochromatic lights, using the same wave-length in centre and surround. Initial reductions followed by a rise in the limen are found with increasing brightness of surround at all wave-lengths, but the reductions are small in the red as compared with the blue end of the spectrum. The effects may be partly due to reflex actions associated with the retinal rods.

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*Cell Organs during Secretion in the Epididymis.\**

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I INTRODUCTION.

Anatomically, the epididymis is the mass of convoluted tubules which connect the testis with the vas deferens. It has been the subject of numerous cytological studies, owing to the fact that the epithelial cells lining its constituent tubules elaborate a secretion, which has been considered to provide nourishment for the spermatozoa during their passage to the vasa. This secretion has been regarded by some observers as of nuclear origin, and by others as a cytoplasmic product.

Benoit has described in cells of the epididymis of certain mammals, including man, the extrusion of nucleolar particles from the nucleus into the cytoplasm.

\* Part of the expenses of this research was defrayed out of a Government Grant of the Royal Society, for which thanks are expressed.

Such particles generally enlarge in the cytoplasm, and are finally ejected into the lumen containing the spermatozoa. The budding-off of portions of the nucleus, containing acidophil granules and karyolymph, was also observed. According to Benoit, therefore, the secretion, which, he considers, subserves a nutritive function for the spermatozoa, is derived principally from the nucleus (1).

Recently Nassonov has published an account of the cytology of the secretory process in the epididymis (11). The secretion, according to this writer, is cytoplasmic in origin, and arises in relationship with the Golgi apparatus. My own work was in progress at the time Nassonov's paper appeared, so that the present paper is devoted to a brief account of Nassonov's work, which I have been able to confirm, together with some additional observations bearing upon the relative rôles of the cell organs during secretion.

Prof. J. P. Hill has kindly read through the manuscript of this paper, and offered valuable suggestions during the course of the work.

## 2 MATERIAL AND TECHNIQUE.

For the greater part of my material I am indebted to Dr. J. A. Murray, Director of the Imperial Cancer Research Fund in whose laboratory part of this work was carried out. The epididymis of the mouse was principally studied. Below is given a summary of the methods used; a full account of the technique has been published elsewhere (7, 8).

### *Summary of Technique Employed for the Study of the Various Cell Organs.*

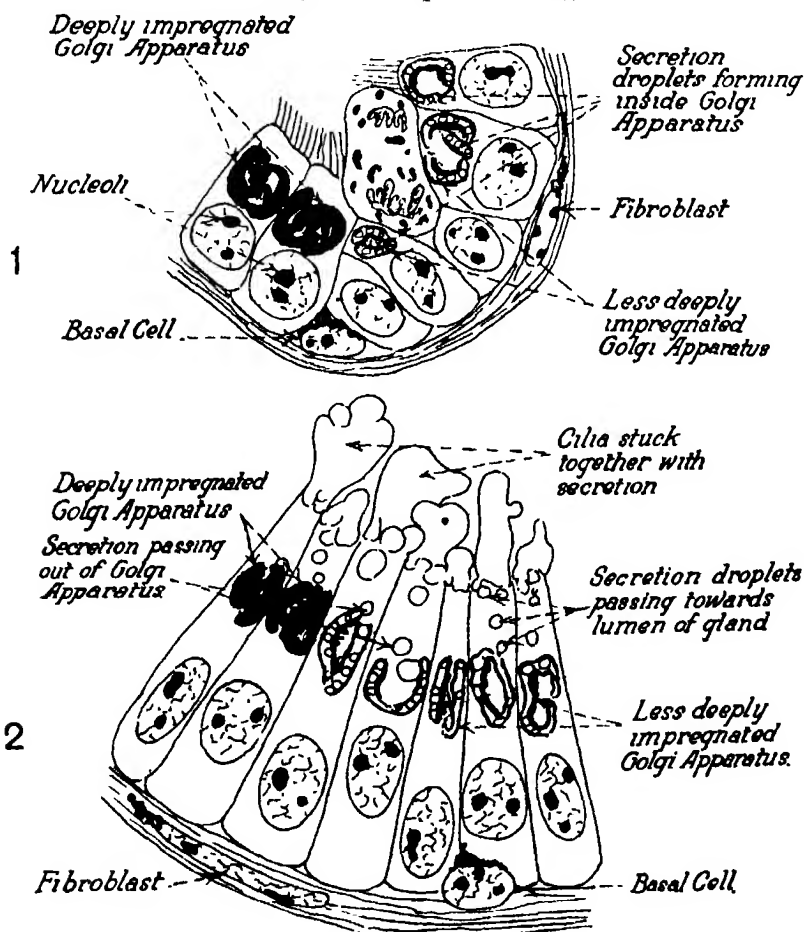
	Fixative.	Stain
For nuclear structures	(i) Bouin's fluid (ii) Boveri's alcohol acetic.	(i) Iron hæmatoxylin, and (ii) Mann's methyl blue eosin.
For Golgi Apparatus.	Modified Kopsch.*	Unstained.
For Golgi Apparatus and Mitochondria.	Modified Kopsch.	Modified Altmann's acid fuchsin.*
For Golgi Apparatus, Mitochondria and Nucleoli.	Modified Kopsch	Altmann's acid fuchsin, differentiated with aurantia, counter- stained with methyl green or toluidine blue.*

\* Full description of the osmic acid methods are given in my paper on "Some Modification of the Osmic Acid Methods in Cytological Technique," 'Jour. R.M.S.,' 1925.

### 3. THE RÔLE OF THE GOLGI APPARATUS DURING SECRETION.

(i) *General*—Important contributions to our knowledge of the part played by the Golgi apparatus in secretion have been made by Cajal, Da Fano, Nassonov, and others. In general, all are agreed that there is hypertrophy of the apparatus at the onset of secretory activity. The greater part of the literature of the subject has been reviewed recently by Nassonov (11)

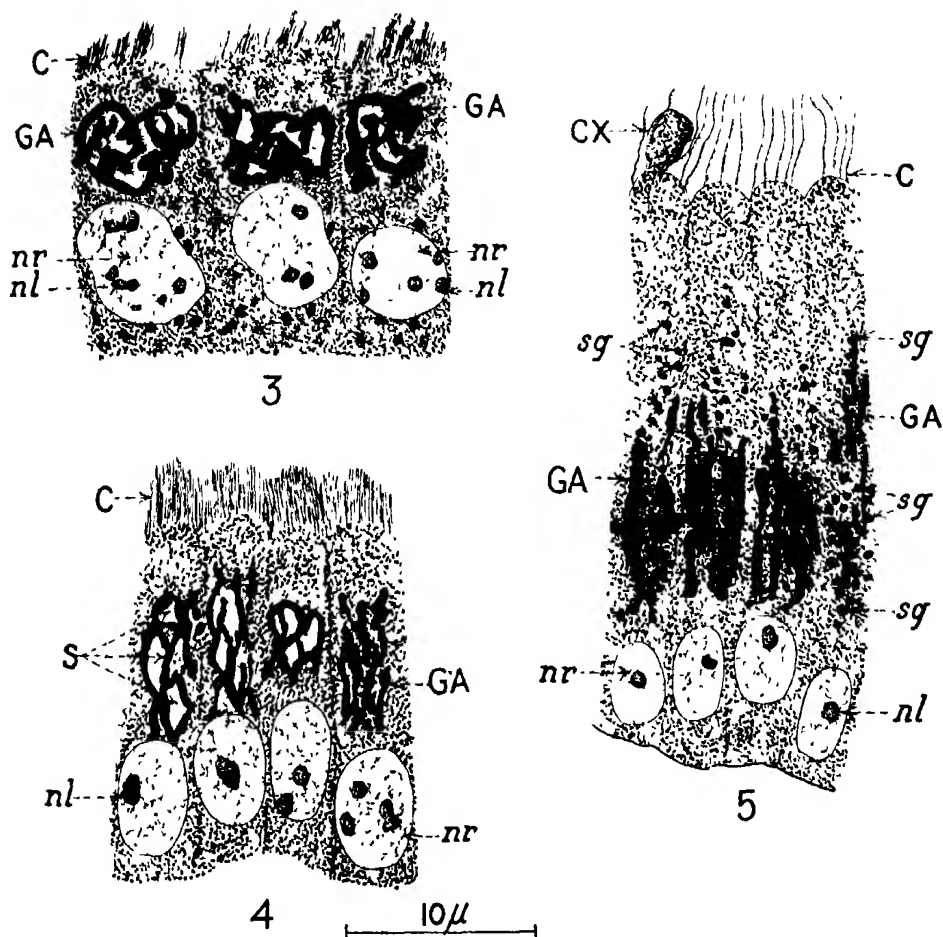
(ii) *The "Liquid" Secretion of the Epididymis.* Droplets of secretion can be seen in the epithelial cells of the epididymis, which have been fixed by any good cytoplasmic fixative. In preparations in which the Golgi apparatus is impregnated these droplets can be seen forming in the substance of the apparatus. Other cells show such droplets passing outwards into the lumen of the epididymis. Nassonov has described and figured this process. Figs. 1 and 2 show two stages



FIGS. 1 AND 2.—Formation of droplets of secretion in the Golgi apparatus of the epithelial cells of the epididymis of the mouse.

in secretion. These figures are reproduced from Nasonov. They are slightly altered, so as to render them more diagrammatic.

Nasonov has pointed out that the structure of the Golgi apparatus varies with the degree of impregnation with the osmium dioxide. The appearances seen, with both deep and slight impregnations, are represented in the first two figures. In figs 3 and 4 are shown camera-lucida drawings of cells in which the apparatus (*GA*) is deeply impregnated. The cells shown in fig 3 correspond



FIGS. 3, 4 AND 5.—Modifications in the form of the Golgi apparatus (*GA*) during secretion in the epithelial cells of the epididymis of the mouse

Fig. 3 shows the deeply impregnated hypertrophied apparatus; fig 4, secretory droplets (*S*) amongst the reticulate apparatus; and fig. 5, secretory "granules" (*sg*) formed under the influence of the apparatus. (Modified Kopsch preparation, unstained.)

to those shown on the left of fig. 1, while those in fig. 4 correspond to similar cells in fig. 2. In fig. 4, secretion (*S*) is seen amongst the ramifications of the apparatus (*GA*). This "liquid" form of secretion is the only secretion described by Nassonov as occurring in the epididymis.

(iii) *The "Granular" Secretion of the Epididymis*—Fuchs described two forms of secretion in the epididymis, a liquid and a granular one (5). Although his work appeared before the recognition of the Golgi apparatus as a definite cell structure, he gave an account of the formation of the two kinds of secretory products in relationship with a canalicular cytoplasmic structure, which undoubtedly was a negative picture of the Golgi apparatus. Nassonov expresses the opinion that the granules described by Fuchs represent modified mitochondria, which in contact with the Golgi apparatus are undergoing transformation into the secretion. My preparations certainly confirm Fuchs' earlier observations. In fig. 5 are shown four cells at the "*caput*" end of the epididymis. It will be noticed, in this figure, that osmophil granules (*sg*) are seen in direct contact with the deeply impregnated Golgi apparatus (*ga*). I regard these granules as distinct secretory products for the following reasons:—

- (1) The mitochondria can be stained separately with acid fuchsin, and I have been unable to distinguish intermediate stages between mitochondria and granules,
- (ii) when sections are bleached with either hydrogen peroxide, or potassium permanganate, the granules retain their black appearance, equally as well as the Golgi apparatus, whereas the mitochondria are rendered quite invisible until the sections are stained;
- (iii) in some parts of the epididymis, where the "liquid" secretion is being elaborated, there is no trace of similar granules

I am, therefore, of the opinion that these granules represent, at least, a different phase of the secretory process, if not a different secretion altogether.

(iv) *The "Complex Granules" of the Epididymis*—Nassonov has described in epithelial cells of the seminal vesicle, the elaboration of complex granules in relationship with the Golgi apparatus (11). One of his figures is represented, somewhat diagrammatically, at fig. 6. According to Nassonov, each of the granules consists of a solid core surrounded by a fluid periphery. Similar complex granules occur in many of my preparations of the epididymis. Figs. 7-10 show typical examples (*X*). The cells figured, by their elongated form, are

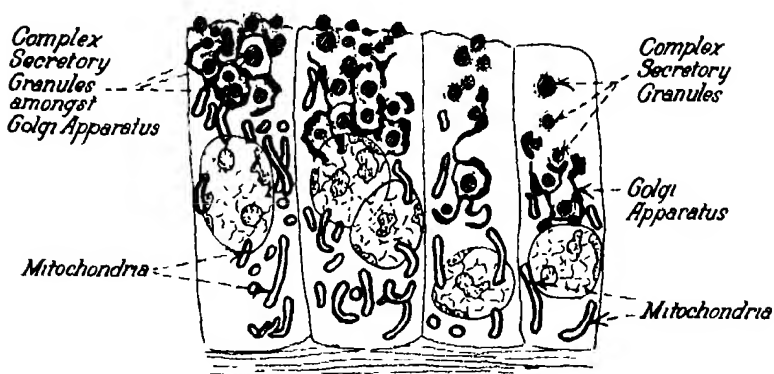
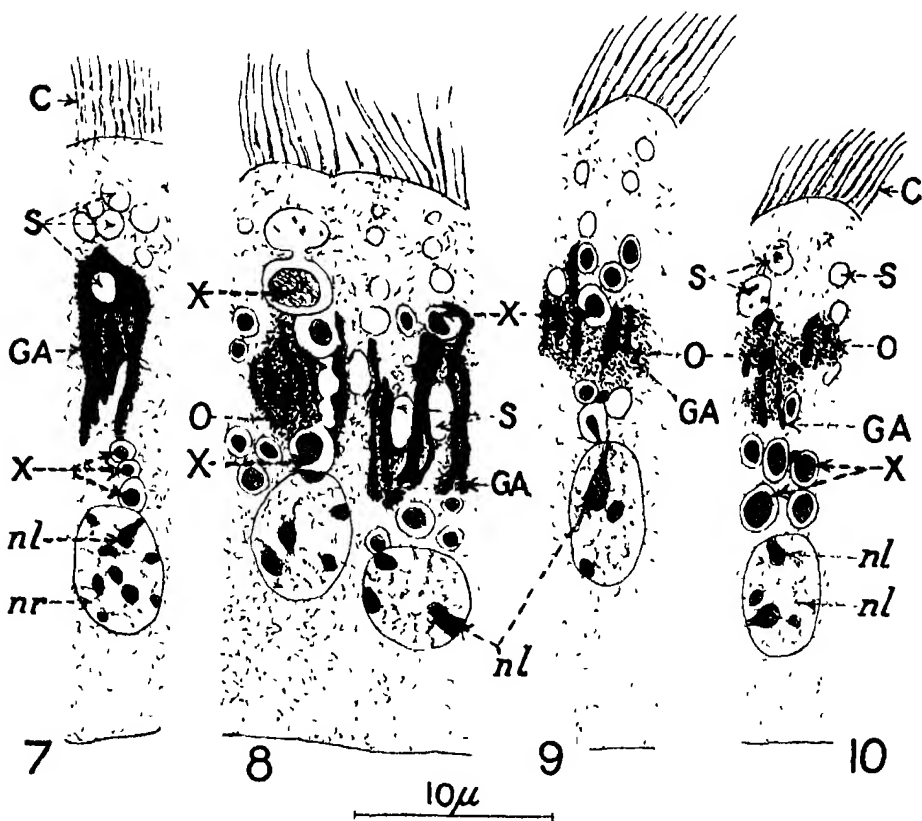


FIG. 6.—Formation of "complex granules" under the influence of the Golgi apparatus in the seminal vesicle of the mouse. Each granule is believed to consist of a solid core surrounded by a fluid periphery (After Nasonov, somewhat diagrammatic.)



FIGS. 7-10 --The complex granular secretions in cells from the "caput" end of the epididymis. (Complex granules (X), nucleolus (nl). (Preparation, modified Kopsch, unstained.)



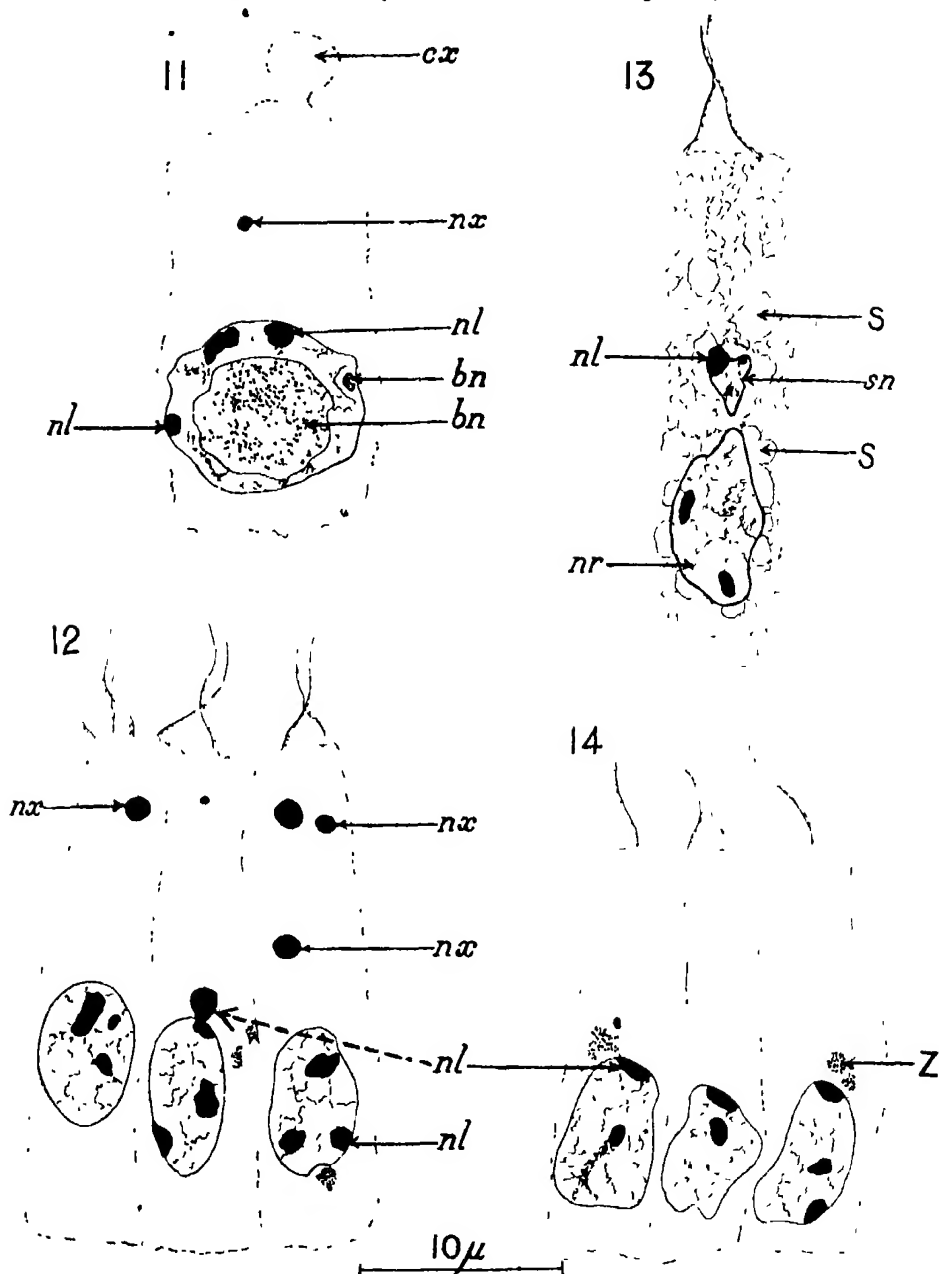
seen to belong to the "*caput*" end of the epididymis. The Golgi apparatus (*GA*) is in the form of elongated threads. The cytoplasm (*O*) immediately surrounding these threads has partly reduced the osmic acid, and in and around this darkened area vacuoles are seen, some of which contain granules (*X*). The clear vacuole-like structures (*S*) are probably identical with the liquid secretion already described, but the question of the origin of the complex granules raises a problem of some difficulty.

In each of the figures 7 to 10, it will be observed that the granules (*X*) occur, not only in and above the region of the cytoplasm occupied by the Golgi apparatus (*GA*), but also between the apparatus and the nucleus. Further, in certain of the cells, especially those shown in figs. 7 and 9, there seems to be a direct relationship between the more solid portion of the granules (*X*) and the nucleoli (*nl*). The questions arise—Are these granules derived from the nucleus, and are they the nucleolar extrusions described previously by Benoit? There is no doubt that we have here a different morphological picture of the secretion. Its significance will be discussed later, after the nuclear processes have been described.

#### 4. THE RÔLE OF THE NUCLEUS DURING SECRETION

(i) *General*—Previous to the study within recent years of the cytoplasmic organs, the part played by the nucleus in various physiological processes was the subject of numerous researches. The changes in the nucleus during secretion have been specially studied by Wace Carlier (12), whose observations have been confirmed by subsequent workers. The general changes observed in the nucleus of the actively secreting cell include a general progressive diminution in the chromatin, and nucleolar extrusions. Benoit, as has been previously mentioned, has described also in the epididymis cells the formation of secondary nuclei.

(ii) *Nucleolar Extrusions in the Cells of the Epididymis.* That the extrusion of portions of the nucleolus from the nucleus into the cytoplasm in cells of the epididymis does occur, there seems little doubt. Figs. 11–14 are drawn from Bouin preparations, stained with iron-alum-haematoxylin. Fig. 12 shows what I believe to be nucleolar extrusions (*nx*). The middle of the three nuclei of this figure shows the extrusion of a part of the nucleolus (*nl*) actually taking place; other granules (*nx*) in the cytoplasm are believed to be derived from the nucleolus. The faintly stained material (*Z*) in fig. 14 is assumed to be of a similar origin.



FIGS. 11-14 Nuclear activity during secretion in cells of the epididymis. Fig. 11, Cell with large secondarily formed nucleolus (*bn*). Fig. 12, Extrusion of nucleolar material from the nucleus into the cytoplasm. Nucleolus (*nl*), nucleolar extrusion (*nx*). Fig. 13, Nuclear budding resulting in the formation of a secondary nucleus (*sn*). Fig. 14, Cells containing granules in their cytoplasm (*Z*), believed to be disintegrating nucleolar extrusions.

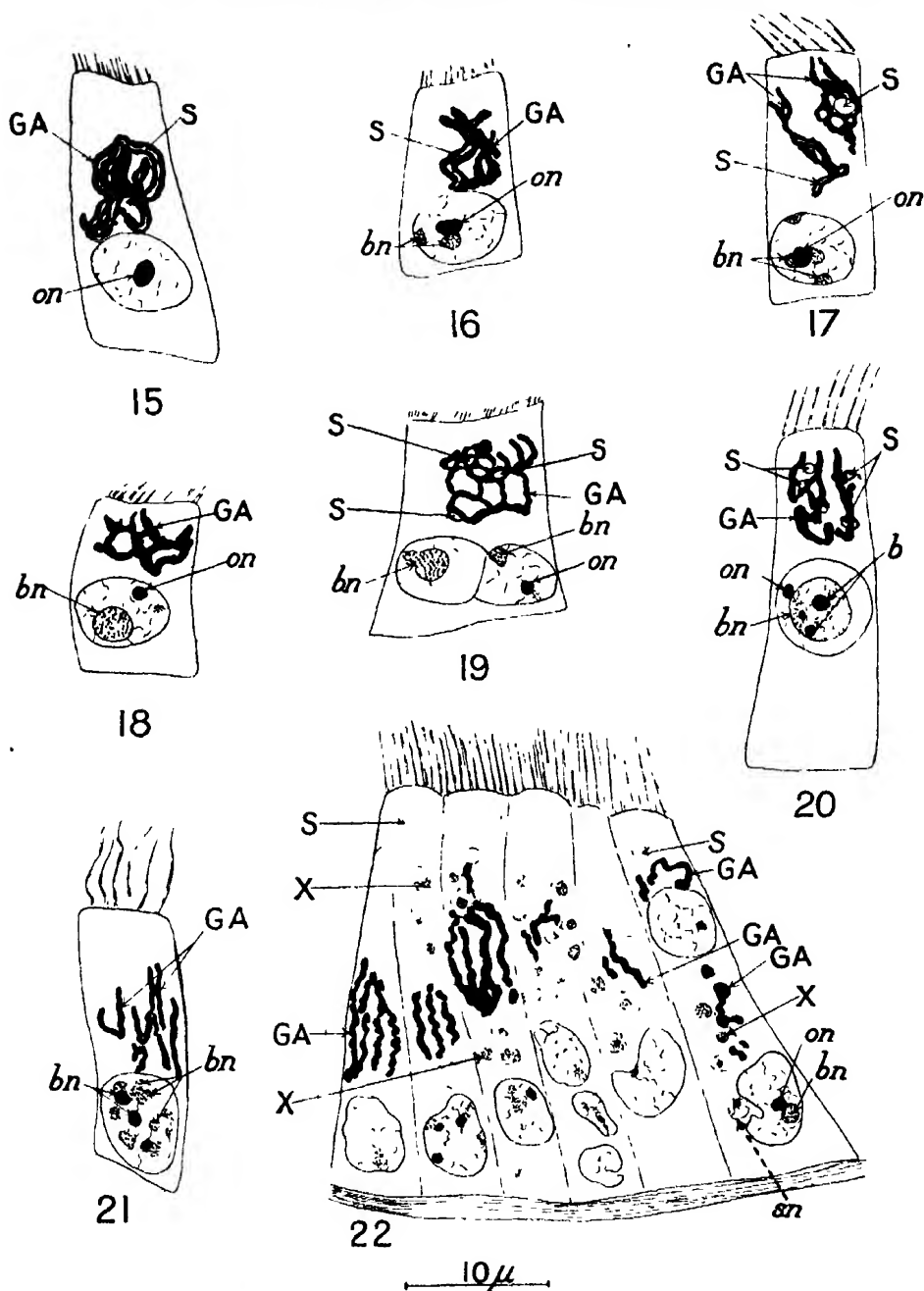
(iii) *The Formation of Nuclear Buds or Secondary Nuclei.*—Bouin-fixed material, suitably stained, also shows indications of the nuclear budding described by Benoit (1). A typical case is shown in fig. 13. The nuclear bud or secondary nucleus (*sn*) is seen to consist of a linin reticulum, with nucleoli (*nl*) similar to the nucleus from which it was derived.

(iv) *Changes in the Nucleolar Content of the Nucleus.*—By employing appropriate technique a differential staining of the nucleolar content of the nuclei can be obtained. In order to compare nuclear changes with changes in the Golgi apparatus, modified Kopsch preparations were partly bleached, and then stained with Altmann's acid fuchsin, which was differentiated with aurantia. Sections were afterwards counterstained with methyl green or toluidine blue. Some nucleoli then stained red, while others were green or blue, according to the stain employed.

In the epididymis cells it is extremely difficult to follow the sequence of changes, compared with what it is in oöcytes during oögenesis. The appearances seen in the epididymis nuclei correspond, however, to the various stages of nucleolar activity, which have been described in the oögenesis of certain molluscs (9, 10). On the basis of this comparison, I have grouped together in figs. 15–22 what I consider to represent the probable course of events in the behaviour of the nucleoli of the cells of the epididymis during secretory activity. In these figures, nucleoli which stain red with the acid fuchsin are shown deep black, while those staining with the toluidine blue or methyl green are shown dotted. After osmic acid fixation, as is well known, the staining reactions of cell contents are often altered, and sometimes completely reversed from acidic to basic, and *vice versa*. As the Golgi apparatus is only visible in osmic treated material, I have adopted throughout this paper the terms oxyphil and basophil nucleoli, according as to whether the nucleoli are stained red with acid fuchsin, or green with methyl green, respectively, in the modified Kopsch preparations.

The probable sequence of changes in nucleolar activity in the cells of the epididymis is as follows:—

- (a) The nucleus contains a single oxyphil nucleolus (*on*) Fig. 15.
- (b) From the oxyphil nucleolus, portions are separated off which undergo a change in chemical composition and stain basophil (*bn*). Figs. 16 and 17.
- (c) The basophil nucleoli increase in size, probably at the expense of the oxyphil nucleolar material, figs. 18, 19 and 20. Sometimes, when a



FIGS. 15-22.—Probable sequence of changes in the behaviour of the nucleolus during secretory activity in cells of the epididymis (Preparation: Modified Kopsch, bleached and stained with Altmann's acid-fuchsin, differentiated with aurantia, and counter-stained with methyl green. Mitochondria are left out of the drawings) The part of the nucleolar content of the cell, which stained oxyphil, is shown deep black (*on*), that which stained basophil, dotted (*bn*)

basophil nucleolus becomes very large, it contains one, or more, deeply staining osmophil granules ("b" in fig. 20). In fig. 11 at "bn" is shown a corresponding stage in Bouin fixed material.

- (d) The basophil nucleoli possibly disintegrate ultimately, and portions may be extruded into the cytoplasm, fig. 21.

It will be noticed from the figures that, starting from fig. 15 and proceeding to fig. 21, there is some sort of orderly change in the Golgi apparatus (*GA*). At fig. 15 the apparatus is tightly coiled, with secretion (*S*) only in its substance. Fig. 16 shows it partly uncoiled, and more so at fig. 17, where there is additional secretion (*S*). At figs. 18 and 19 the apparatus has a more open appearance, while figs. 20 and 21 show it approaching towards the elongated threadlike form which is characteristic of the most active stage of secretory activity, as shown in figs. 7-10.

The cells shown in figs. 15-22 are not all taken from the same part of the epididymis. Those shown at figs. 15 to 19 are from the middle of the epididymus, the remainder, 20 to 22, from the "*caput*" end, so that it is questionable to what extent we are justified in accepting the scheme, represented in these figures, as consecutive stages in the morphological aspect of the secretory process. However, this is justifiable if, as is suggested, different appearances seen at various parts of the epididymis are due to differences in the intensity of the secretory activity. This aspect of the problem will be discussed later.

In considering the differential staining of the nucleolus, the question arises as to which part of the nucleolus it is that is extruded into the cytoplasm. In fig. 22 is shown the formation of a secondary nucleus, and also various bodies marked "X" which are believed to be derived from the nucleolus. The nuclear bud, or secondary nucleus, contains an oxyphil nucleolus, and some of the granules "X" have an oxyphil core, but most of them are basophil. Three stages in nucleolar extrusion are shown also in fig. 24, where the extruded material is apparently basophil ( $nx_1$  and  $nx_2$ ), and a similar process is represented in fig. 25, but in the latter figure one of the granules is partially oxyphil. In other parts of the epididymis the extruded nucleolar material is certainly originally oxyphil, and becomes basophil later. It, therefore, seems that both types of nucleolar material are extruded into the cytoplasm, the basophil material preponderating at the most active stages of secretion. Even when oxyphil material is discharged it soon becomes basophil, so that it may be that when it is basophil material that is discharged, the chemical change, upon which the basophil staining reaction depends, has taken place inside the nuclear membrane instead of in the cytoplasm.

## 5. THE RÔLE OF MITOCHONDRIA DURING SECRETION.

(i) *General.* E. V. Cowdry (4) in his valuable paper on "The Mitochondrial Constituent of Cytoplasm" has pointed out how various workers have attributed a mitochondrial origin to practically all the specialized substances formed by cells. His criticism of this tendency applies specially to secretion, where often mitochondria have been said to transform into substances of a totally different chemical constitution. Nasonov has expressed the opinion that the mitochondria may give rise to intermediate products, which in contact with the Golgi apparatus, and under its influence, are changed into the final secretion granules. Bowen (2) believes that the Golgi apparatus is responsible for the formation of enzymes which control the elaboration of the secretion. The relative rôle of these two categories of cell organs is very difficult to decide.

(ii) *Mitochondria during Secretion in the Epididymis* —Nasonov has described and figured changes in the mitochondria of the cells of the epididymis during secretory activity. One of his figures, slightly more diagrammatic, is reproduced at fig. 23. The mitochondria, together with the other cytoplasmic inclusions, are represented in figs. 24 and 25.

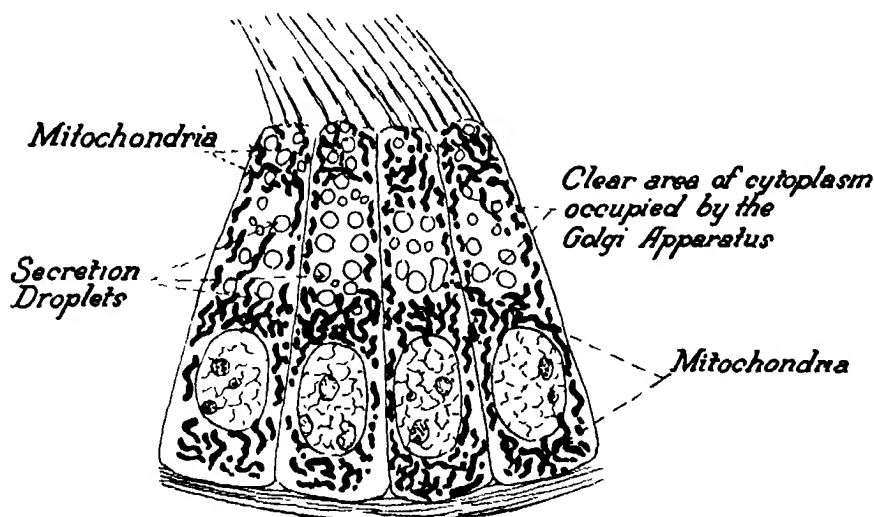
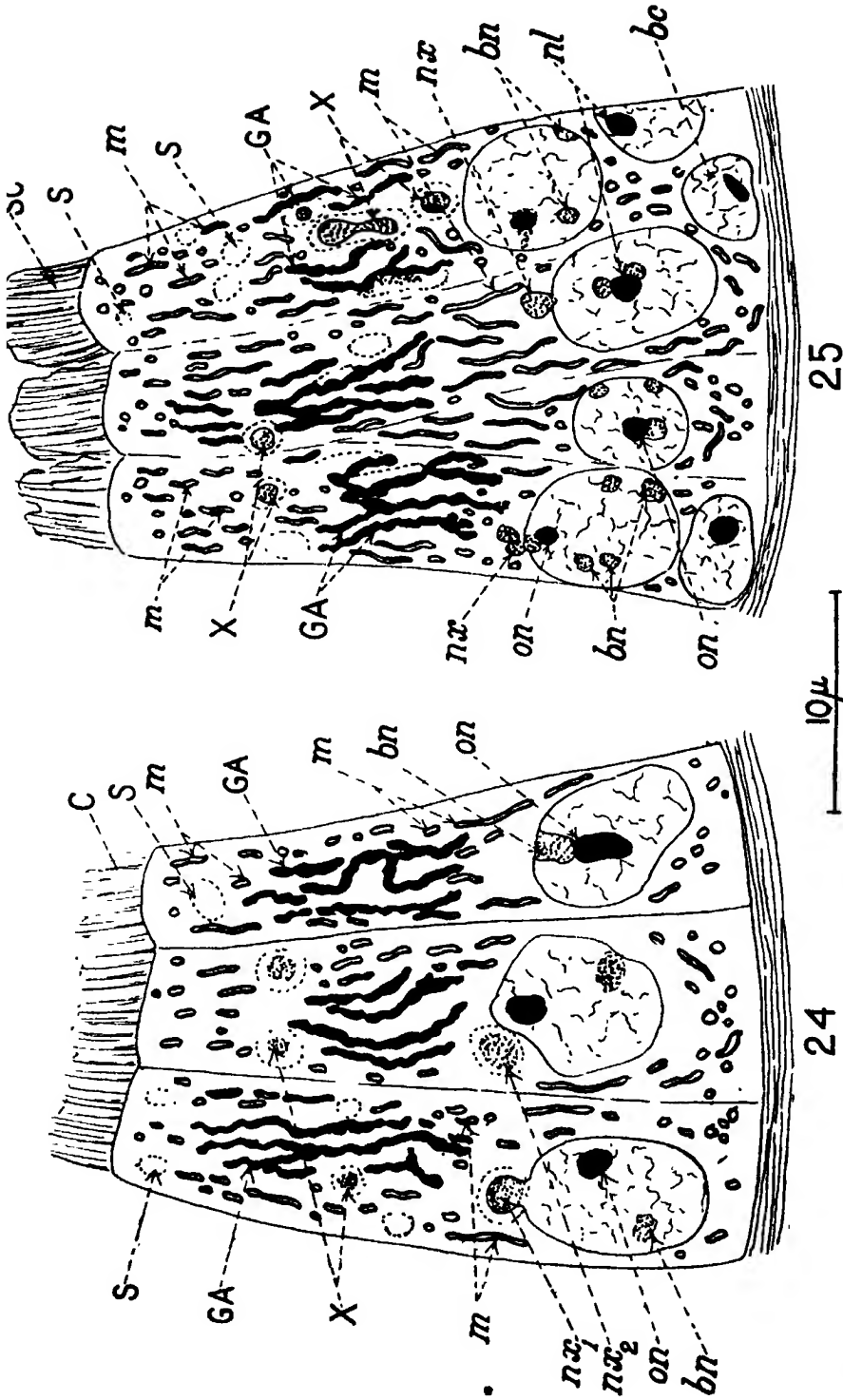


FIG. 23 — Mitochondria in cells of the epididymis.

At the onset of secretion the mitochondria increase in numbers, and there is a diminution after secretory activity, so that they contribute to the production of formed bodies seems practically certain, but the exact nature of their contribution is a matter for theoretical discussion rather than practical demonstration.

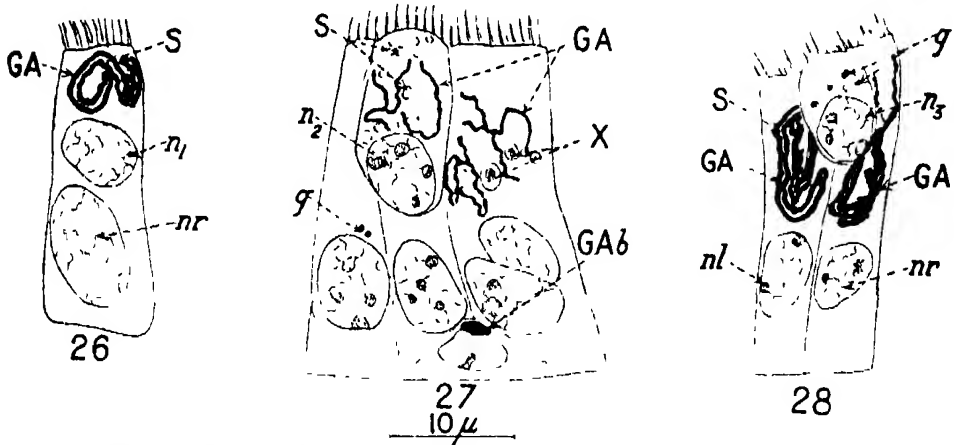


Figs 24-25.—The complete cytological structure of cells of the epididymus during secretion. Golgi apparatus ( $GA$ ); mitochondria ( $m$ ); nucleolar extrusions ( $nx$ ). In fig 24 are shown three stages in nucleolar extrusion—“ $bn$ ,” “ $nx_1$ ,” and “ $nx_2$ ,” (Preparation same as figs 15-22.)

## 6. THE MAINTENANCE OF SECRETORY ACTIVITY IN THE EPIDIDYMIS.

(i) *The Discarding of Exhausted Cells.*—Throughout the greater part of the epididymis the formation of secretion droplets seems to proceed somewhat rhythmically, and at a relatively slow rate. Pauses between periods of heightened activity probably allow the cells time to recover. In certain tubules, however, especially towards the "*caput*" end, secretory activity is very intense. The Golgi apparatus becomes altered in form, with reduction in size; the nucleus becomes smaller, with depletion in chromatinic contents, and the mitochondria are diminished in numbers. Possibly the nuclear buds which give rise to secondary nuclei, and the nucleolar extrusions, are in part the means whereby the nucleus eliminates its waste products. The material extruded from the nucleus may serve as raw material for the elaboration of the secretion.

Amitosis is another possible means of getting rid of waste nuclear material. Figs 26-28 show what are considered to be stages in this process. In fig. 26



FIGS 26-28—Stages in what is regarded as the process of elimination of waste products following amitosis. Fig. 26—cell with two nuclei believed to have been formed as the result of amitosis. Figs 27 and 28—show what are assumed to be stages ( $n_1$  and  $n_2$ ) in the discharge of cells such as may be constituted by the upper nucleus " $n_1$ " of fig. 26, and a portion of the cytoplasm of the same cell. (Preparation—modified Kopsch, unstained.)

two nuclei are shown in the cell. From comparison with other cells, these nuclei are assumed to have originated as the result of amitotic nuclear division. The upper of the two nuclei ( $n_1$ ), it is believed, may become separated from the lower one, and, together with a portion of the cytoplasm, constitute a cell such as is shown in fig. 27, with its nucleus marked " $n_2$ ". This cell contains



a Golgi apparatus and secretion droplets (*S*). The cell with the nucleus ( $n_3$ ) in fig. 28 shows what is regarded as a stage later. In this cell, instead of a Golgi apparatus, there are osmophil granules (*g*), which are considered to have originated from the disintegration of the apparatus. Cells presenting similar characters to this have been observed in the lumen of the tubules of the epididymis, so that it is suggested that disorganized cells such as the one shown in fig. 28 ( $n_3$ ) may be cast off into the lumen of the tubules, though I have not been able to observe cells actually being expelled in the manner described.

F. W. R. Brambell (3) has recently described in epithelial cells of the oviduct of the fowl, the re-formation of a Golgi apparatus after its dissociation, concurrently with secretory activity. Brambell believes that the Golgi apparatus is formed anew in the cell during its period of recovery. Certain granules appear near the nucleus, and these are the first indication of the reconstruction of the apparatus. Possibly something of the same kind occurs in the epithelial cells of the epididymis. In the cell on the extreme left of fig. 27 are seen two granules (*g*) which are probably of a similar origin to those described by Brambell, the original apparatus of this cell having been separated off into the cell above ( $n_2$ ). These granules may represent a portion of the apparatus left behind when the separation of the two cells occurred. The cell at the extreme right of fig. 22 supports this latter view.

In none of the cells of the epididymis is the Golgi apparatus actually discharged with the secretion. After intense secretory activity, granules or rodlets represent the remains of the apparatus, as is shown in figs. 5, 9 and 10. There is usually an osmophil zone of cytoplasm (*O* in the figures) around such granules (see especially figs. 8-10). It is in this region of the cytoplasm that the new apparatus is reconstructed.

(ii) *The Replacement of Discarded Cells* — If exhausted cells are discharged into the lumen of the epididymis there must be some means for replacing them. Two possibilities suggest themselves here :—

- (a) The remaining cells may divide and fill up the gaps.
- (b) The basal cells (*bc*) may replace the exhausted cells ; fig. 25.

The latter I believe to be the usual procedure, for these reasons :—

- (a) The basal cells (*bc*) vary in size from cells with flattened nuclei to cells with their nuclei bigger and rounder ; compare figs. 1 and 2.
- (b) Mitosis has been observed in these basal cells.
- (c) The basal cells are usually larger where exhausted cells are being discharged into the lumen (see "*GAb*," fig. 27).

## 7. THE RELATIVE RÔLES OF THE CELL ORGANS DURING SECRETION.

There has been a tendency amongst many cytologists, who have studied changes occurring in cells under different conditions of metabolism, to limit their attention to either nuclear or cytoplasmic phenomena. The result has been that some have attributed to the nucleus the principal rôle, while others have regarded the mitochondria or Golgi apparatus as of chief importance. J. Bronté Gatenby, in his valuable series of papers on "The Cytoplasmic Inclusions of the Germ Cells" (6), while primarily concerned with the study of mitochondria and Golgi apparatus during gametogenesis, has emphasised the importance of nuclear phenomena. The behaviour of the cell organs during oögenesis, described by Gatenby and other workers, resembles in some respects the changes described as occurring in secreting cells of the epididymis. During oögenesis in certain animals (*e.g.*, *Saccocirrus*), as described by Gatenby, and during secretion in the epididymis there occur :—

- (a) Nucleolar extrusions.
- (b) An initial increase in the number of mitochondria.
- (c) Increase, or hypertrophy, of the Golgi apparatus.

Undoubtedly, as in oögenesis, so in secretion, each of the cell organs contributes its part. The cell as a whole is a functioning unit, and we are probably approaching nearest to the truth in studying the changes occurring in its visible structure, rather than seeking to attribute substances formed by it to the activities of any one of its component parts.

It is problematical how far the differences seen in the cells of various tubules of the epididymis are due to varying intensities of the secretory process, rather than being indicative of the formation of different secretions. The essential morphological changes to be observed in the cells of the epididymis during secretion are as follows :—

- (a) A portion of the nuclear content is extruded into the cytoplasm, either in the form of nucleolar extrusions, or as nuclear buds, forming secondary nuclei, both of which finally diffuse into the cytoplasm, or under the influence of the Golgi apparatus become directly converted into secretion ;
- (b) the Golgi apparatus becomes hypertrophied, and secretion droplets, or granules, make their appearance in intimate relationship with it ;
- (c) there is an initial increase in the mitochondrial content of the cell, and many mitochondria are seen to be in immediate contact with secretory products, *see* figs. 24 and 25.

The following scheme represents variations in these processes suggested as occurring at varying degrees of secretory activity :—

- A. *Secretion Slow*.—Few or no spermatozoa in tubule. Secretion in the form of granules (Fig. 5.)  
These granules diffuse into the cytoplasm, and portions of it (CX) become budded off into the lumen of the tubule. Nucleolar extrusion, but not well marked.
- B. *Secretion Rapid*—Numerous spermatozoa in tubule. Secretion appears in the form of droplets and “complex granules.” Extensive nucleolar extrusion and nuclear budding. (Figs. 22, 24, 25.)
- C. *Secretion Intermediate*.—Numerous spermatozoa in tubule. Secretion in the form of droplets. Nucleolar extrusions. (Figs. 3 and 4.)

#### 8. SUMMARY.

1. *During secretory activity in the epithelial cells lining the tubules of the epididymis, the following morphological changes are to be observed in the cell organs :—*

(A) *Golgi Apparatus*.—This hypertrophies, and assumes different forms according to the degree of secretory activity. The secretory products which make their appearance in intimate relationship with the apparatus are of three types :—

- (i) “droplets” (figs. 1-4),
- (ii) “granules” (sg, fig. 5),
- (iii) “complex granules,” probably consisting of a solid core and a fluid periphery (X, figs. 7-10)

(B) *Nucleus*—There occur :—

- (i) nucleolar extrusions (nx, figs. 12, 24 and 25),
- (ii) nuclear budding, resulting in the formation of secondary nuclei (sn, figs. 13 and 22),
- (iii) differential staining of the nucleolus. The following is the probable sequence of changes occurring during secretion :—
  - (a) there is a single oxyphil nucleolus (on, fig. 15),
  - (b) part of this becomes basophil (bn, fig. 16), and increases in size at the expense of the oxyphil part (on, fig. 16). See figs. 17-20,

(c) finally the basophil nucleolus breaks up, and portions are extruded into the cytoplasm (*bn*, figs. 21 and 22).

(The terms oxyphil and basophil refer to the staining reactions in osmic-fixed material.)

(C) *Mitochondria.* - Increase in number at the onset of secretory activity, and decrease during the course of secretion. Often they are found in immediate contact with the formed secretion (*m*, figs. 24 and 25).

2 *It is suggested that variations observed in the secreting cells, in different tubules of the epididymis, may be indicative of variations in the degree of intensity of the secretory process (see Summary, page 370)*

3. *Secretory activity in the cells of the epididymis is maintained by—*

(A) *The elimination of waste products of metabolic activity* This is effected by :—

(i) nucleolar extrusions (*nx*, figs. 12, 24 and 25) and nuclear budding (*sn*, figs. 13 and 22). The product of such nuclear activity may, under the influence of the Golgi apparatus, contribute to the formation of the secretion.

(ii) Amitosis (fig 26) followed by the separating apart of a portion of the protoplasm so as to constitute a small cell. Such cells (figs. 27,  $n_2$ , and fig. 28,  $n_3$ ) are believed to be cast off into the lumen of the tubule

(B) *By the reconstruction of exhausted cells.*—After intense secretory activity the Golgi apparatus may be represented by deeply impregnated granules (*g*, figs. 27 and 28. *GA*, fig. 22), or by an osmophil zone in the cytoplasm (*O*, figs. 8–10), or sometimes both. A new apparatus is reconstructed about such centres.

The chromatin of the nucleus and the mitochondrial content of the cells are at the same time restored to the normal.

(C) *By the replacement of worn-out cells.*—This is believed to be brought about in the following ways :—

(i) Cells which have become completely worn-out are replaced by the basal cells (figs. 1, 2, 25 and 27).

On page 368 are set out the reasons for this assumption.

(ii) There may be also some replacement of exhausted cells, as the result of the division of neighbouring ones, but mitosis is extremely rare.

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## EXPLANATION OF LETTERING OF FIGURES.

- b* —Intra-nucleolar granules impregnated by osmic acid.  
*bc* —Basal cell.  
*bn*.—Basophil-staining nucleolus.  
*C*.—Cilia.  
*CX*.—Portion of cytoplasm, containing secretion, being extruded into lumen of tubule.  
*g*.—Granules believed to represent remains of the Golgi apparatus after intense secretory activity.  
*GA*.—Golgi apparatus.  
*GAb*.—Golgi apparatus of basal cell.  
*m*.—Mitochondria.  
*n*.—Nucleus.  
*nl*.—Nucleolus.  
*nr*.—Nuclear reticulum.  
*nz*.—Nucleolar extrusion.  
*O*.—Osmophil zone of cytoplasm.  
*on*.—Oxyphil staining nucleolus.  
*S*.—Secretion in the form of "droplets."  
*sj*.—Secretion in the form of granules.  
*sn*.—Secondary nucleus.  
*X*.—Granules believed to be partly nucleolar in origin.  
*Z*.—Granules probably representing disintegrating nucleolar extrusions.

*The Relation between the Development, Structure and Functioning of the Nodules on Vicia faba, as influenced by the Presence or Absence of Boron in the Nutrient Medium.*

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[PLATES 27-30.]

The problems connected with the nodules that are produced by *Bacillus radicicola* on leguminous plants fall naturally into four divisions :

- (a) The life of the organism in the soil and the causes leading to its arrival at the usual point of infection—the root hair.
- (b) The entry of the organisms into the tissues of the plant.
- (c) The causes leading to the formation and development of the nodules after the entry of the organism into the plant.
- (d) The physiological processes within the nodule, especially those leading to the fixation of elemental nitrogen.

The life of the organism outside the plant and the causes leading to its initial entry into the tissues do not concern the present paper, which deals with the relation between the organisms within the nodule, and the anatomy and physiology of the tissues of the host plant.

The general course of formation of a nodule is well known and has been carefully described by other workers (6, 20, 29, 31). The entry of the bacteria results in a complicated response on the part of the plant, whereby, under normal conditions, there is produced an outgrowth having, in its centre, a mass of swollen cells containing large numbers of the organisms. In the developed nodule almost the whole of the organisms are to be found in these swollen cells. Since nitrogen fixation has been shown to be associated with the presence of the bacteria (12), it is most probable that this swollen cell tissue with its large numbers of contained organisms is the seat of this process. The central problems in the physiology of the nodule, therefore, are the causes leading to the formation of this swollen cell tissue and the nutrition of the bacteria within it.

With regard to the nutrition of the bacteria, it is an important point that

they require a free supply of energy-giving material. Numerous experiments with other nitrogen-fixing bacteria have shown that a large supply of easily oxidisable organic material is necessary for nitrogen fixation (3, 13, 34), and carbohydrates and related substances have proved to be the most efficient sources of energy for this purpose (15). Both on theoretical grounds and by analogy with organisms fixing nitrogen in culture, therefore, one may suppose that the bacteria within the nodule require an abundant supply of carbohydrate or similar easily available compounds of high energy value.

Under normal conditions vascular strands develop which run up the sides of the nodule \* A consideration of the requirements of the bacteria for nitrogen fixation suggests that these strands have the function of supplying them with carbohydrate as a source of energy and of removing the products of their metabolism. On this view the vascular supply forms, physiologically, the connecting link between the plant and the bacteria within the nodule. A study of the exact relation between the strands and the development and functioning of the nodule thus becomes a first essential to any understanding of the physiology of the organisms within it.

Hitherto it has not been possible to study the rôle of this vascular supply by comparing the growth and metabolism of normal vascular nodules with that of nodules where the vascular supply was deficient or absent. The abnormal growth produced by growing the broad-bean (*Vicia faba*) in water culture from which boron has been excluded and, in particular, the derangement of the vascular development in the root produced in this way, provide a means whereby this problem may be attacked.

The important rôle of boron in the nutrition of the broad-bean has recently been clearly demonstrated by Warington (32), though the physiological function of the element still remains obscure. Further experiments have entirely confirmed her results, and have also revealed a definite relationship between the presence of boron and the structure and development of nodules on the roots.

#### A.—*Origin of the Work.*

The investigation arose as a result of some observations made on sand cultures of *Vicia faba* grown in the presence and absence of boron. With boron 13, 38, 65 and 146 nodules were developed on the individual plants; whereas of the plants in the boron-free environment, one had nine nodules and the remainder none. In this experiment the nodules were due to chance infection, no bacterial inoculum being intentionally added. The result, how-

\* These were observed by Tréviranus in 1853 (25).

ever, stimulated an investigation into the effect of a total absence of boron on nodule formation, cultures in nutrient solution being employed as giving better controlled conditions.

B.—*Smaller Number of Visible Nodules developed in a Boron-free Medium.*

*Experiment I.*—A series of broad beans was grown in a mineral salt solution\* of which the reaction was adjusted to give a pH value of 6.2, this being favourable to the activity of *Bacillus radicola*. Half the plants received boron at the rate of 1 : 500,000 boric acid, and all were inoculated by introducing into each bottle at every change of solution a few c.c. of a culture of *B. radicola* grown in Prucha's "medium 335" (21) with the agar omitted, the liquid medium being more convenient to handle. On plants supplied with boron, nodule formation became evident after about a month, and numbers of typical, good-sized nodules were produced. In the absence of boron, the first visible signs of nodule formation were delayed for another month and the nodules remained small and undeveloped throughout.

The number of nodules visible on individual plants† in the two series were as follows :—

Table I.

<i>With Boron.</i>	<i>Without Boron.</i>
234	116
248	147
351	201
420	241
512	288
—	—
Mean 353	199

*Experiment II.*—Tests were made to determine how nodule formation was affected by the *absence* of boron in solutions of varying acidity and also by the absence of nitrate, as many authors (22, 27, 28, 33) have shown that *excess*

\* Potassium nitrate, 1 grm.; magnesium sulphate, 0.5 grm.; sodium chloride, 0.5 grm.; calcium sulphate, 0.5 grm.; potassium di-hydrogen phosphate, 0.3 grm.; potassium mono-hydrogen phosphate, 0.27 grm.; ferric chloride, 0.04 grm.; distilled water to make up 1 litre.

† In the experiments here described, the number of nodules given refers, of course, to those attaining macroscopic size. As mentioned below, there is reason to think that, on roots grown without boron, a number of nodules are formed, but cease to grow before they become visible.



of nitrate reduces nodule production. In this case the first inoculation was delayed until the plants were well started.

The results of this experiment are shown in Tables II and III.

Table II.—Inoculated Broad Beans. July 18–August 28, 1923. Average of 5 plants. With Nitrate in Solution.

Reaction of Medium.	No Boron					With Boron.				
	Shoot.	Root.	Total.	Average number of Nodules	Range	Shoot	Root	Total	Average number of Nodules	Range.
	Grm	Grm.	Grm			Grm	Grm.	Grm		
pH 3.8	2.452	0.300	2.752	—	—	4.544	0.744	5.288	98	10–206
pH 5.0	2.426	0.360	2.786	—	—	4.022	0.632	4.654	113	2–367
pH 6.2	2.332	0.318	2.650	17	86 (on one plant only)	4.882	0.724	5.606	210	17–402

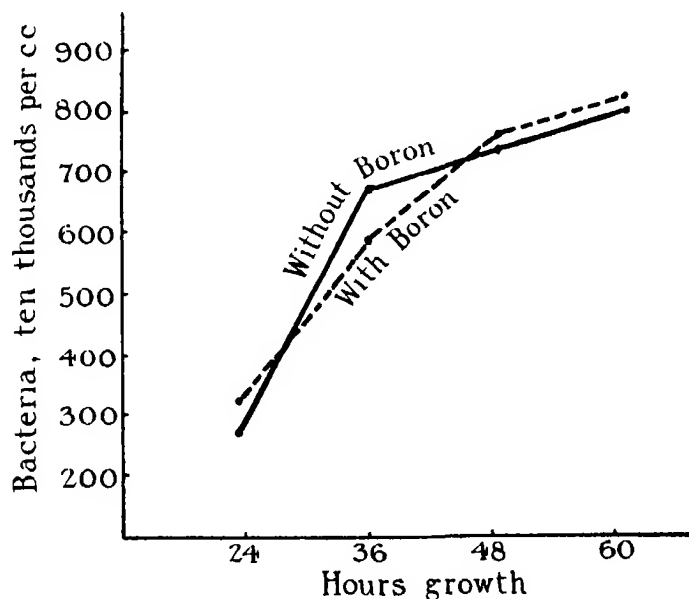
Table III.—Inoculated Broad Beans. July 18–September 20, 1923. Average of 5 plants. Without Nitrate in solution.

Reaction of Medium	No Boron				With Boron.			
	Shoot.	Root	Total	Number of Nodules	Shoot.	Root	Total	Number of Nodules
	Grm	Grm	Grm		Grm.	Grm.	Grm	
pH 3.8	1.195	0.211	1.406	—	1.365	0.465	1.830	—
pH 5.0	1.252	0.305	1.557	—	1.360	0.465	1.825	—
pH 6.2	2.498	0.525	3.033	71 (range 8–205)	2.046	1.213	5.259	673 (range 334–990)

It will be seen that in cultures supplied with nitrate, the presence of boron enabled nodules to develop even at a H-ion concentration of 3.8, while in the absence of boron no visible nodules were produced at the higher acidities. Thus the unfavourable effects of acidity on nodule formation were increased by the absence of boron. In the absence of nitrate no nodules were visible at the higher acidities either in the presence or absence of boron. At the more favourable reaction of pH 6.2, nodules were developed both with and without nitrate, though in the latter case more nodules were seen. At this reaction the decreased number of visible nodules in the absence of boron, noted in Experiment I, is again striking.

C.—Effect of Boron on *Bacillus Radicicola* in Pure Culture.

The last two experiments having shown that the number of visible nodules was reduced in the absence of boron from the culture solution, the question arose as to whether the presence of traces of boron was necessary for the proper growth and activity of the nodule organism in this solution. In order to investigate this point, the growth of the organism in culture solution in the presence and absence of boron was studied. For this purpose, Prucha's "medium 335" (21), made up without agar,\* was used. Both the components and the solution itself were tested for traces of boron with negative results. The same strain of organisms as was used for inoculation throughout the work was grown in this solution, without boron and also with one part of boric acid to 500,000 of solution. The cultures were grown in test-tubes each containing 10 c.c. of solution, inoculated with a suspension of bacteria from a two days old culture. The number of organisms in this suspension was counted on a hæmocytemeter and each tube was inoculated with 0.1 c.c. of the suspension, so diluted that this volume contained approximately 60,000 bacterial cells. The tubes were incubated at 25° C. At intervals, counts of the number of organisms in each medium were made as follows:—A standard loopful of the

FIG. 1.—Growth of *Bacillus radicicola* from *Vicia faba*.

\*  $K_2HPO_4$ , 0.2 grm.;  $MgSO_4 \cdot 7H_2O$ , 0.2 grm.;  $NaCl$ , 0.2 grm.;  $CaSO_4$ , 0.2 grm.;  $CaCO_3$ , 0.2 grm.; saccharose, 20 grms.; tap water, 1 litre.

suspension from a tube was placed on a Thoma hæmocytometer, the organisms were killed with osmic-acid vapour and the number of cells on 30-50 squares was counted. The process was repeated with four separate loopfuls, and from the mean of the counts obtained the number of organisms per cubic centimetre was calculated. On each occasion the mean count from three parallel tubes was taken. The rate of multiplication of the organisms with and without boron is shown in fig. 1, where the number of organisms in the cultures is plotted against the time of incubation. It will be seen that the presence of boron in no way affects the multiplication of the bacteria.

The nodule organism passes through very definite changes in morphology, and to ascertain whether the absence of boron produced any effect on this cycle of changes, preparations stained with carbol fuchsin were made and examined at each time of counting. It was found that the changes through which the organism passed were similar in the presence and in the absence of boron.\* The evidence from growth in pure culture, therefore, provides no reason for supposing that the absence of boron from culture solution surrounding the bean roots has any harmful effects on the nodule organism suspended therein.

*D.—Cultural Evidence that the Absence of Boron from the Solution bathing the Roots is not Harmful to Infection.*

Assuming, therefore, that the absence of boron is not harmful to the normal activity of the nodule organism outside the host plant, there appear to be two possibilities left. Either the presence of traces of boron in the solution surrounding the roots at the time of infection is beneficial to the entry of the organism into the tissues, or the plant growth and physiology induced by the absence of boron is unfavourable to nodule development, and in order to decide between these possibilities, the following experiment was made —

*Experiment III.*—Broad beans were grown with and without boron for

\* Juliu Voicu (1923), 'Influence du Bore sur quelques bactéries du sol' (Edition de la vie Universitaire, Paris), tested the effect of varying doses of boric acid on growth and nitrogen fixation of *Bacillus radicicola* in a medium consisting of haricot-bean extract to which nitrogen compounds and saccharose were added. The medium used as control already contained 0.005 mgrm. of boron per cent. He states: "On ne constate aucun effet stimulant sur le *Bacillus radicicola* de la vesce; mais, une légère action toxique pour la dose de 10 mg. de bore %. Le microbe des nodosités du pois est nettement favorisé par les doses fortes de 5 à 10 mg. de bore % (0.3 à 0.6 gr. d'acide borique par litre)." At these comparatively strong concentrations a stronger growth and fixation of nitrogen was found to occur. At concentrations of boric acid of the order employed by the present authors, Voicu found no effect on the organisms.

17 days. Three methods of inoculation with *B. radiculicola* were then adopted; first, plants grown without boron and having abnormal roots were inoculated in a solution containing boron; secondly, plants grown in a solution containing boron and having normal roots were inoculated in the absence of boron; and, lastly, plants were both grown and inoculated in the presence of boron. After eight hours' contact with the organisms the roots were well washed in running water\* and replaced in fresh supplies of solutions similar to those in which they were first grown.

The number of nodules that developed are shown in Table IV

Table IV.—Relative Effect of Root-Type and Inoculating Solution on Nodule Formation.

Numbers of Visible Nodules on Individual Plants.

Series A. Grown <i>without</i> boron Inoculated in the <i>presence</i> of boron.		Series B Grown <i>with</i> boron Inoculated in <i>absence</i> of boron		Series C. Grown <i>with</i> boron Inoculated in the <i>presence</i> of boron	
	252		649		543
	122		638		498
	105		576		488
	26		334		440
	6		294		384
Mean	102		498		422

It will be seen that on plants with abnormal roots grown without boron comparatively few nodules developed, even though boron was present when the plants were immersed in the inoculating fluid. The nodules on these plants were small, and tended to be sunk in the tissues. On plants with normal roots grown with boron many more nodules were developed, and their numbers were unaffected by the presence or absence of boron in the inoculating solution. On these normal roots the nodules were well developed, in contrast to those on the abnormal roots. This experiment thus indicated that the absence of boron from the surrounding medium does not affect the entry of the bacteria into the tissue, but rather that growth of the plant without boron has affected the development of the nodules.

\* It is probable that some organisms escaped being washed off the roots, but that the bulk of the infection occurred during the immersion in the inoculating suspension.

*E.—Nitrogen Fixation in the Absence of Boron.*

*Experiment IV.*—The above and similar experiments suggested the question as to whether the nodules that do form are normal, and able to fix nitrogen effectively. Cultures of broad beans were, therefore, set up in an inoculated solution containing no nitrate with the addition of—

- (a) No boron.
- (b) 1 : 2,500,000 boric acid.
- (c) 1 : 500,000    ,,    ,,
- (d) 1 : 100,000    ,,    ,,

The seeds were graded in weight, and a sample analysed for nitrogen, the actual nitrogen content\* varying from 0·05432 to 0·05626 grm. N, averaging 0·05529 grm. N per seed. The initial growth was good, but less rapid than in a parallel series receiving nitrate. Nodules appeared first within 17 days on plants with boron, but were a few days later in its absence. From this time onwards the difference between the plants with and without boron became increasingly marked.

*No Boron.*—These plants, on which the nodules presented a curious burst appearance, rapidly fell behind those receiving boron, and after a few weeks showed signs of nitrogen starvation as well as a lack of boron, as the leaves began to turn yellow from the bottom upwards, at the same time that the typical death of the growing point, upper leaves and flower buds was occurring.

*With Boron.*—The beans grew well, remaining green to the end, and producing good roots with fine laterals, covered with numerous well-developed nodules, the average numbers per plant being 675, 695, and 784 in the three series. Although they grew more slowly than similar ones receiving nitrate, they were apparently not suffering from nitrogen starvation. It appeared that nitrogen derived from the air dissolved in the food solution had been made available to the plant through the activity of the nodule organisms, which were, therefore, functioning normally.

Without boron the yellowing of the leaves indicated acute nitrogen starvation after the initial supply stored up in the seed had been utilised, in spite of the fact that a considerable number of nodules had been produced. The inference was that in the absence of boron the nodules were unable to function properly, either because the organism was inhibited from making use of the atmospheric nitrogen or because the nitrogen compounds formed by the organisms were in

\* We are indebted to Mr. G. C. Sawyer for the nitrogen determinations in this experiment.

some way prevented from gaining access to the plant. The truth of this hypothesis was proved by determination of the nitrogen content of each individual plant, the actual amount of nitrogen fixed by the nodules being obtained by subtracting the average amount of nitrogen initially present in an average seed from the total quantity in the grown plant. The total nitrogen fixed per plant was ten or twelve times as great with boron as without, proving clearly the greater efficiency of the nodule when supplied with boron. The increased efficiency is not simply due to an increase in number, but to a definitely greater activity of the individual nodules, as is seen by comparing the amounts of nitrogen fixed per nodule with the different treatments.

Table V - Nitrogen Fixation. Average of 5 Plants.

A Treatment	B Percentage N in Dry		C. Total N fixed per Plant *	D. Number of Nodules	E. N fixed per Nodule †
	Shoot	Root.			
			Grm		Grm.
No boron, including green plant‡	2 46	2 60	0 026	491	0 0000536
No boron, excluding green plant	2 32	2 59	0 016	455	0 0000343
1 . 2,500,000 boron acid	3 43	3 06	0 182	675	0 000269
1 . 500,000 " "	3 35	2 91	0 165	695	0 000238
1 . 100,000 " "	3 27	2 78	0 148	784	0 000188

\* In addition to the average 0 05529 grm. N supplied by the seed.

† Obtained by dividing column C by D

‡ This plant remained greener and more healthy than the rest, possibly owing to a higher boron content in the seed, which delayed the appearance of the characteristic phenomena due to boron deficiency.

The greater efficiency in the presence of boron is still more clearly shown when the comparative activity of the nodules on each individual plant is compared. This may be determined by dividing the average amount of nitrogen fixed per nodule on each plant by the smallest average amount fixed per nodule on any plant in the series.

For example :—

Smallest average amount of N fixed by any nodule .....	= a
Actual average amount of N fixed per nodule on plant under consideration.....	= b
Comparative activity of nodules .....	= $b/a = x$ .

The comparative activity ( $x$ ) of the nodules with no boron ranged from 1.0 to 18.83, with an average of 8.38. If the one exceptional plant which remained green is excluded, the range is 1.0 to 10.63, the average falling to 5.77. Where boron was present, the range was from 21.30 to 69.97, the averages being 48.04, 41.60, 34.82, with increasing concentrations of boric acid.

\* Table VI.—Comparative Activity of Nodules in Nitrogen Fixation.

Plant No	No Boron	1 · 2,500,000 Boric Acid.	1 · 500,000 Boric Acid	1 · 100,000 Boric Acid.
1	3 13	44 00	36 52	26 36
2	18 83	69 97	59 62	21 30
3	8 33	49 87	31 00	36 58
4	10 63	30 65	44 45	61 62
5	1 00	45 72	36 43	28 26
Average	8 38	48·04	41 60	34 82
„ excluding 2	5 77	—	—	—

From the above table it is evident that in the absence of boron the functioning of the nodule is seriously interfered with and the activity of the organisms impaired.

#### F.—*Effect of Boron on Structure of Nodule.*

The results already detailed led directly to an examination of the effect of boron on the structure of the nodule. Nodules were obtained from the plants grown in Experiments III and IV, providing material subjected to considerable variation of treatment both as regards boron supply and general nutrition. The material was fixed in acetic acid and corrosive sublimate, embedded by Dowson's (8) rapid method of paraffin infiltration and stained with gentian violet and vesuvian brown.

A fully developed nodule, under normal conditions, consists mainly of a mass of large, thin-walled, nucleated cells, more or less completely filled with bacteria.\* Towards the apex, on the side farthest removed from the middle of the root, may be a number of fairly large, rather empty cells, in which the bacteria have not as yet multiplied to any great extent, and beyond this again is a small-celled meristem in a state of active division. The nodule is surrounded by an endodermis, which is continuous across the base, but which appears

\* For convenience this tissue is hereafter termed the "bacteroidal tissue," many of the bacteria contained in it being in the form of bacteroids. The term "tissue bactéroïdien" is due to Tschirch (26).

to be interrupted across the meristematic tip. Between the endodermis and the bacteroidal tissue are several layers of tissue which are traversed by vascular strands given off from one or more points opposite the protoxylem in the vascular cylinder of the root. Very frequently two strands pass off from the same protoxylem group at different levels, and in large nodules additional strands may be given off from one or more adjacent protoxylem groups. (See also Eriksson (9) and Vuillemin (29)\* ) These consist of vascular elements surrounded by nucleated cells of which many are rather densely protoplasmic. The strands run the full length of the nodule, and merge into the meristematic tissue, the smaller protoplasmic cells being distinguishable for a rather greater distance than are the lignified elements. The strands follow a sinuous course and often branch, and in cross section as many as ten may appear, some of which in reality represent the same strand cut through more than once owing to the curvature. No evidence of anastomosis has been obtained, even in large nodules with an abundant vascular supply. Each strand is surrounded by an endodermis which is continuous with that of the root, and which is not evident quite so far as the point at which the tissues merge into the meristem (9, 29). Nodules of this type are characteristic of every case in which boron has been supplied to the plant, though the number and degree of branching of the vascular strands appear to depend to a great extent upon the size and vigour of the nodule. On inoculated plants supplied with a nutrient solution containing an adequate supply of nitrate the nodules are comparatively large, and often show more than two strands, with a considerable degree of branching. On similar plants not supplied with nitrate it rarely happens that more than one or two strands are produced, and these do not branch so freely, but in both cases the strands develop until they extend the full length of the nodule, and an abundance of healthy bacteroidal tissue is found. (See text-fig. 2)

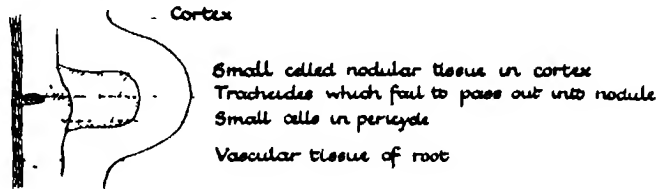
In the absence of boron such nodules are very rare, being replaced by incompletely developed nodules of two main types (*see* text-fig. 2). In the extreme case the nodule is abortive or undeveloped (Type I). Cell proliferation occurs in the inner layers of the cortex, but no obvious boundary layer is formed round the groups of tissue. On the inner side the cells in the region of the endodermis often suffer some changes or degeneration, developing into a more or less protuberant layer strongly marked by a mass of deeply staining, disorganised substance, which may arise from the breaking down of cells.

\* The origin and structure of vascular strands in the normal nodule have been carefully described by Vuillemin (29). The observations of the present authors agree in general with his description.

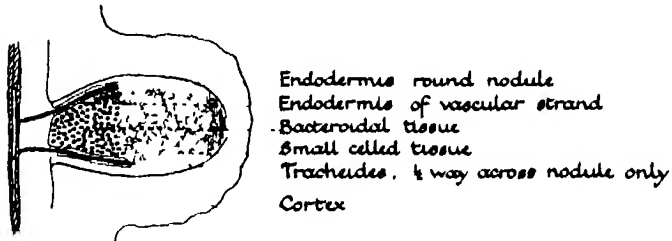


Internal to this a certain amount of cell proliferation occurs in the pericycle, causing a slight swelling, and a very few tracheides may or may not be present, but they do not penetrate through into the incipient nodular tissue in the cortex

### No Boron. Type I Abortive or undeveloped nodule



### Type II Incompletely developed nodule



### With Boron. Fully developed nodule

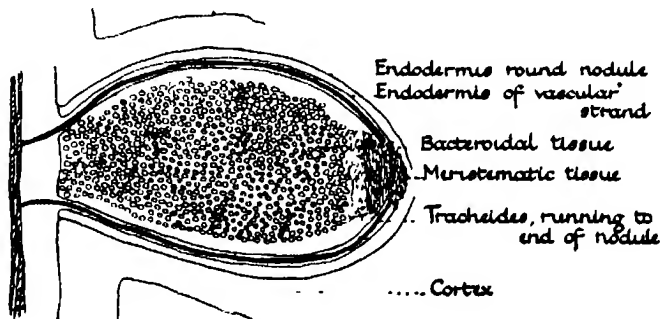


FIG. 2.—Diagrams showing influence of boron on development of nodules of *Vicia faba*. (Diagrams are compounded from observations of numerous series of sections, the sinuous vascular strands being projected into one plane.)

(text-fig. 3). It is very easy to overlook nodules of this type when examining roots macroscopically, as they are entirely buried in the cortex and make but little external swelling, rendering it almost impossible to distinguish them from incipient lateral roots, even if they are detected. This may account for

the small number of nodules usually counted on roots grown without boron. It is likely that in these cases a considerable number of nodules fail to reach macroscopic size, and that reduced nodule growth rather than diminished infection of the root is the explanation of the low nodule counts.

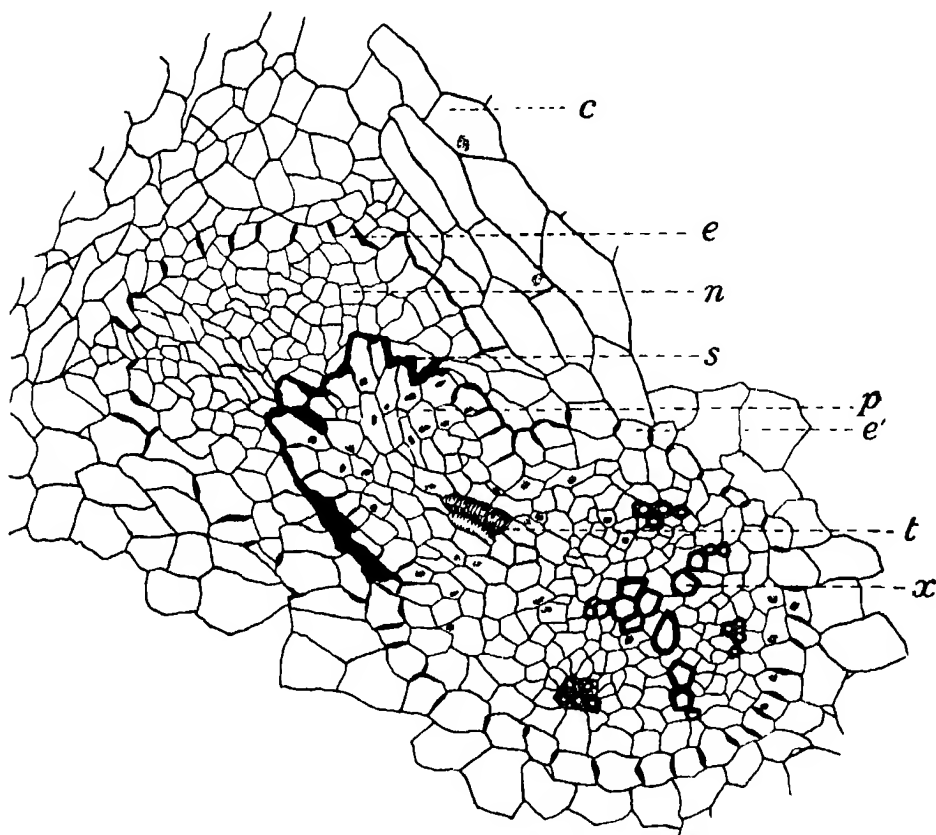


FIG. 3 —Transverse section of root of *Vicia faba* grown without boron, showing abortive nodule with no vascular strands or bacteroidal tissue.

*e*, endodermis of nodule. *e'*, endodermis of root. *n*, undifferentiated nodular tissue in cortex. *p*, proliferated cells in pericycle. *s*, deeply staining sheath between pericycle and nodular tissue. *t*, tracheides, not passing out into nodule. *c*, cortex. *x*, xylem of root.

The other type of nodule formed in the absence of boron develops further but incompletely (text-fig. 2, Type II). The bacteroidal tissue, instead of occupying the bulk of the nodule, is confined to a more or less constricted space at the inner end, the rest of the nodule being filled with rather smaller cells which would apparently have developed into bacteroidal tissue under more favourable conditions. Furthermore, the bacteroidal tissue is frequently

abnormal in character, being either undeveloped or in a state of apparent disintegration as regards the cell contents. The vascular strands are rarely more than one or two in number, they branch very little, and, more significant than all, they seldom run the full length of the nodule, but usually extend only about as far out as the bacteroidal tissue, or slightly beyond it. This immediately suggests that some intimate connection exists between the development of the vascular strands and the bacteroidal tissue, and an attempt has been made to obtain some statistical estimate of this association. A number of nodules grown with and without boron under various conditions have been examined section by section and an estimate made of the percentage of bacteroidal tissue present, and of the length of the vascular strands relative to that of the nodule. Altogether 72 nodules from plants supplied with boron and 87 nodules from plants deprived of boron have been worked through. Throwing the percentage of bacteroidal tissue into frequency curves (text-fig. 4), it is seen that in the presence of boron 80-100 per cent. is most usually attained, less than 40 per cent. being observed in but one

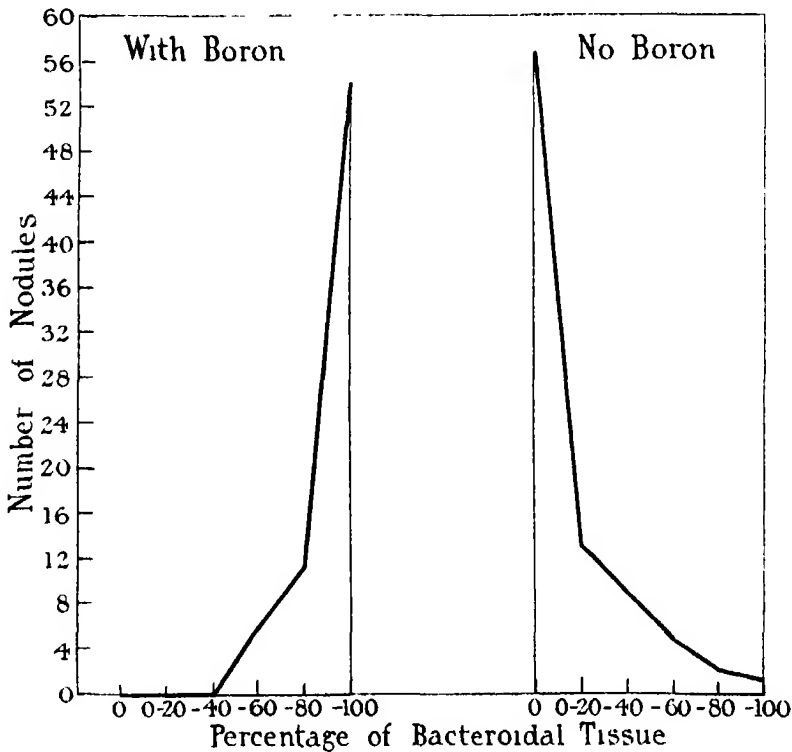


FIG. 4.—Curves showing the relative proportions of nodules with low and high percentages of bacteroidal tissue in the presence and absence of boron in the nutrient medium.

instance. In the absence of boron, on the other hand, an entire absence of bacteroidal tissue is most usual, and only in very exceptional cases does the percentage rise above 60. Even these few cases of high percentage may be explained by the fact that most of them occurred on plants which were not supplied with any nitrate, so that growth was slow and the boron contained in the seed was, therefore, not used up very quickly, and it is quite conceivable

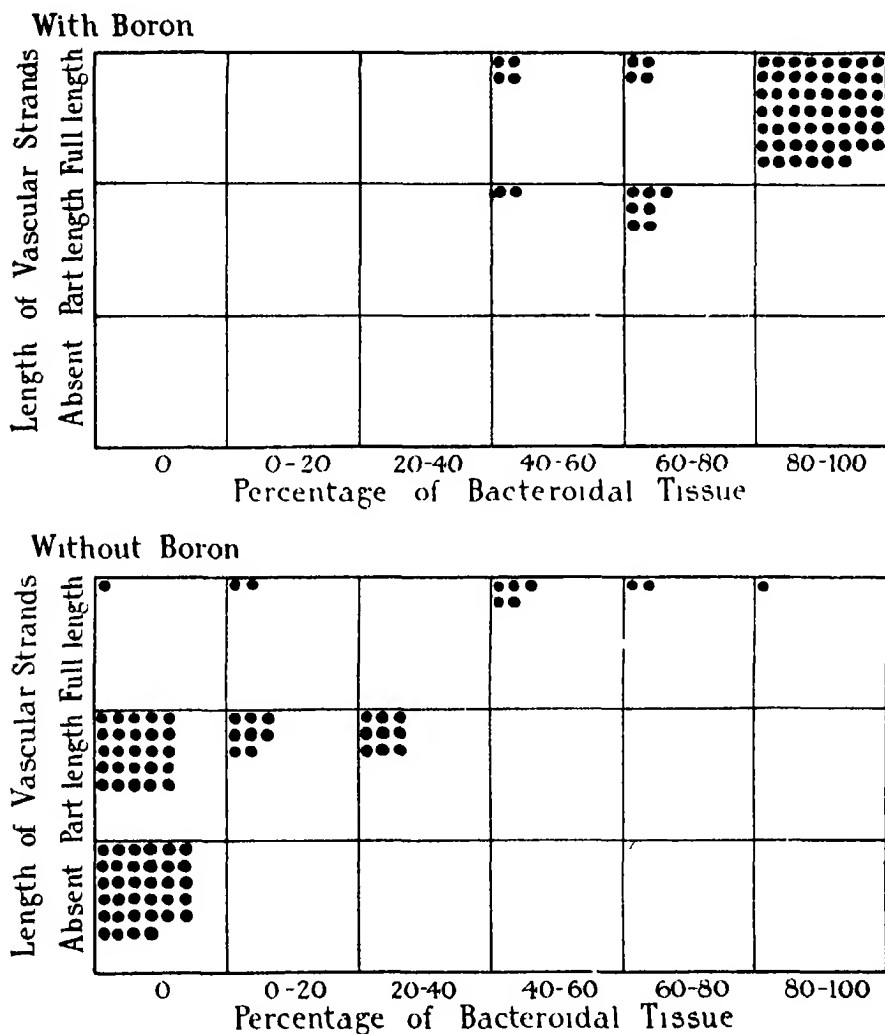


FIG. 5.—Showing relation between length of vascular strands in regard to the nodule and proportion of bacteroidal tissue developed. Each dot represents one nodule observed.

Total number of nodules with boron = 71.

Total number of nodules without boron = 87.

that part of this reserve boron supply aided in the better development of some of the earlier formed nodules on these plants.

Further analyses of the figures indicate that the production of bacteroidal tissue follows closely in the train of the development of the vascular strands, and that where the latter are arrested in their progress from any cause, the bacteroidal tissue suffers a corresponding diminution. The two charts (text-fig. 5) show graphically how close is the association between the development of vascular strands and the proportion of bacteroidal tissue. In the presence of boron the bulk of the nodules show strands running the full length, with a high percentage of bacteroidal tissue, shorter strands and less bacteroidal tissue being to all appearance connected with younger nodules not yet fully grown. In the absence of boron, on the other hand, the majority of the nodules show no bacteroidal tissue, even with a partially developed vascular system, and in the cases where a certain proportion of such tissue does occur, it is usually abnormal in type and in a state of degeneration. Occasionally, the strands run in advance of the bacteroidal tissue, so that a three-quarter or full-length strand may be associated with less than 30 per cent. bacteroidal tissue or none, instead of with 80 per cent. or more. The reverse, however, does not hold good, as no single instance has been observed in which a large proportion of bacteroidal tissue was associated with a shortened vascular strand.

In the normal nodule, as in the nodule grown without boron, the development of bacteroidal tissue bears an evident association with the growth of the vascular strands (Plate 28, figs. 7, 8, and 9). This tissue does not extend beyond the extremity of the strands and may lag slightly behind them. Its development also takes place earlier at the sides of the nodule than in the centre, where the cells are at a greater distance from the conducting tissue (Plate 28, fig. 8).

G.—*Investigation of the Effect of the Vascular Supply on the Bacteria within the Nodule.*

A study of the general morphology of nodules, grown in the presence and in the absence of boron, thus establishes a relationship between the development of the conducting tissue of the nodule and the formation of the fully developed swollen cell tissue containing large numbers of "bacteroids." Furthermore, analysis shows that in plants grown in the absence of boron, where the conducting tissue of the nodules is absent or deficient, there is little or no fixation of nitrogen. The bacteria in these nodules are thus not acting normally with regard to their nitrogen metabolism, nor do the tissues of the plant react normally to the stimuli produced by infection. It seemed probable, therefore,

that a careful study of the progress of infection and of the behaviour of the bacteria within these abnormal nodules would lead to a better understanding of the factors at work in the normal nodule.

Our present knowledge of the development and structure of nodules on leguminous plants is mainly derived from researches conducted during the nineteenth century. At the time of these investigations the cycle of change in morphology through which the nodule organisms pass was not appreciated. Various different forms of the organisms, such as the swollen "bacteroids," the rod forms and the motile "swarmers," had been seen\* by various authors, but it was not until the appearance of Löhnis and Smith's paper in 1916 (16) that the existence of a definite life-cycle was appreciated. Bewley and Hutchinson (2), however, in 1920 fully described the changes through which the organisms pass in artificial media, and suggested that these changes formed a definite life-cycle. This cycle is of importance in the present connection because certain stages in this life-cycle are also found in the tissues of the nodule. These were observed by Wallin [(30), *see also* de Rossi (23)], and the observation is corroborated by the present authors.

In investigating the course of development of nodules under normal conditions and in the absence of boron, therefore, it was desired to study the effects of the two sets of conditions: first, on the life-cycle of the bacteria within the nodule; and, secondly, on the action of the organisms upon the cells of the host plant.

(a) *Technique*.—The material used in this portion of the work was derived from Experiments III and IV, in which plants were grown with and without nitrate and in the presence and absence of boron. Two fixatives were used, Bouin's picro-formol (4) and Flemming's stronger mixture (10). The fixative was washed out in 70 per cent. alcohol, and after dehydration was embedded by Dowson's method (8). Sections 5 m. thick were cut on a Litz-Minot microtome. Many methods of staining were tried in an attempt to get a good differentiation of the bacteria. The best results were obtained by staining the sections with carbol fuchsin, washing rapidly in 30, 50, 70, and 90 per cent. alcohol, and counterstaining with lichtgrün dissolved to saturation in 90 per cent. alcohol. For staining bacteria within the infection threads, it was found that a saturated solution of bengal rose in 5 per cent. phenol, replacing the carbol fuchsin in the above method, gave some very fine preparations, in which the bacteria were stained red and the matrix of the infection thread green. This method, however, was very uncertain in its results, the bengal

\* Some references to these earlier observations are given by Löhnis, 1921 (14).

rose being easily washed out in alcohol. Iron hæmatoxylin, followed by lichtgrün, was used where the cytology of the tissues was being specially studied.

(b) *Development of the Normal Nodule*—The course of development of the normal nodule, in the presence of boron, was first followed, in order to have a basis for comparison with that of the abnormal types having defective vascular strands. In the normal root, the piercing of the cortex by the infection thread is followed by a multiplication of the cortical cells immediately adjoining the endodermis. This cell proliferation is limited to the inner tissue, the more external cells of the cortex do not divide. The multiplication of cells produces a mass of small-celled tissue which may be described as the "primary nodule" (Plate 28, fig. 5).

In this the infection thread ramifies, entering the cells in all directions. Within the cell the infection thread sometimes passes along the primordial utricle and sometimes crosses the middle of the cell. Strands containing only a single row of bacterial rods can frequently be seen crossing from the cell wall to the neighbourhood of the nucleus (Plate 27, fig. 1), and it seems probable that in such cases the bacteria have made use of the cytoplasmic strands of the host cell as a path of infection. In any case, the frequency with which the infection threads run up to the nucleus, as described by previous authors, was very noticeable in our preparations.

The bacteria in the infection thread are always in the form of minute rods whose protoplasm stains uniformly with carbol fuchsine or bengal rose (*cf.* Maria Dawson (6)). They are enclosed in clear spaces resembling capsules, which do not take up any of the stains used. These lie in a matrix which stains readily with lichtgrün. In several cases the infection thread has been seen running into cells with nuclei in various stages of mitosis, but this is rare, and it is clear that infection of a cell as a rule arrests its division.

After the formation by cell division of the primary nodule, further development is associated with the outgrowth of the vascular system from the stele. Young nodules, in which the strands had not yet grown into the nodule, were found showing an incipient swelling of the cells close to the stele. The swelling of the cells thus begins at the base of the nodule, and as the vascular strands grow it proceeds in an outward direction. By this time cell division in the mass of the primary nodule has been arrested by the infection of the majority of cells with the ramifying bacterial threads. At the distal extremity of the nodule tissue, however, the cells remain meristematic, and by rapid division produce an apical growing point (Plate 28, fig. 6). The bacterial strands

now grow outward towards the newly formed tissue thus produced, continually infecting fresh cells. In this way the secondary growth in length of the nodule is produced, and, in such nodules, all the stages in the infection and swelling of the cells can be seen in different parts of a longitudinal section. Close behind the apical meristem are young cells that are protoplasmic, having only small vacuoles. The infection threads can be seen entering these (Plate 30, fig. 20). The cells close behind this show the early results of infection on the cells. The uninfected cells in this region swell and become vacuolated, so that they resemble normal parenchyma cells. The infected cells also become enlarged and vacuolated and at first do not differ largely from the uninfected cells.

Some of the bacteria now pass out from the infection thread into the protoplasmic lining of the cell wall. The bacteria, after their release, lie singly in the cytoplasm of the host. They consist of evenly staining rods which could not be shown to possess capsules by any staining method tried (Plate 27, fig. 2). They very quickly multiply, become longer and exhibit a segregation of the staining material into bands crossing the rods. Increase in the bacterial numbers is accompanied by a further increase in size of the host cell and by a thickening of its protoplasmic lining. The nucleus increases in size, usually remaining in its normal position in the cell, and only in very old infected cells does it appear to be degenerating. When the infected cells are fully developed, the bacteria lying in the thickened protoplasmic lining have increased enormously in number and show a striking change in morphology. They become swollen and vacuolated, and stain faintly with fuchsine. These swollen forms, the "bacteroids" of Brunchorst (5) are, as is well known, characteristic of the "bacteroidal" tissue of the well-developed normal nodule. In this tissue one frequently finds remains of the infection threads crossing the swollen cells. These threads still contain bacteria. It is a point of some interest that the bacteria in these infection threads always remain in the form of minute uniformly staining rods, as described above (Plate 27, fig. 3). The factors in the environment that so greatly modify the bacteria lying free in the cytoplasm of the host do not effect any change in the appearance of the bacteria lying within the infection thread in the same cell.

(c) *Infection and Nodule Development in the Absence of Boron.*—In plants grown in the absence of boron the early development of the nodule is normal. The organisms enter the root hair (Plate 29, fig. 10), the infection thread, containing short rod forms, penetrates the cortex (Plate 29, fig. 11) and cell division takes place in the tissue adjacent to the endodermis and results in the



formation of the "primary nodule" (Plate 29, fig. 12). The course of events from this point differs according to the presence or absence of vascular strands in the nodule, and the extent of their growth where present.

In a great number of nodules produced in the absence of boron no vascular strands are developed at all (Type I, p. 384). A mass of cells is formed by multiplication of the tissue adjoining the endodermis, but from this stage development of the nodule is abnormal, no secondary growth being made. The conditions in such nodules are very interesting. The infection thread, which contains rods surrounded by clear spaces as in normal nodules (Plate 27, fig. 4), ramifies through the cells and, probably owing to its action in stopping cell division, the nodule soon reaches a condition in which no dividing cells can be found (Plate 29, figs. 13, 14), because the meristem cap is either not formed or, if produced, soon becomes invaded with bacteria, as described below. The cells in the centre of this arrested "primary nodule" swell somewhat in size and become vacuolated. The infection threads in this tissue undergo a remarkable development. Expansions and lobate processes appear on them (Plate 30, fig. 20). Swellings can be seen on infection threads in normal nodules and have been described and figured by previous authors (20, 24), but in such cases they are comparatively small. In the present case they develop to so great an extent as finally to fill up the whole cell, crushing the nucleus into one corner, and by further growth they form masses of bacterial zooglœa, which break up the nodule tissue. In Plate 29, fig. 15, a nodule is shown having a considerable portion of its centre destroyed and filled up with bacterial mass. The bacteria in these nodules never become swollen in the normal manner, but exist as rods or cocci. In most cases they seem to remain embedded in a zooglœal mass, but this is not always so, for they are sometimes seen lying free in the cells. It is an interesting point that the apical meristem of the nodule, if formed, is usually attacked by the bacteria, the small protoplasmic cells becoming filled with masses of rods and cocci. This is shown in Plate 30, fig. 19, which should be compared with the section of meristem in a normal nodule (Plate 30, fig. 20).

Where there is a partial development of the vascular strands (Type II, p. 384) the swelling of the infected cells takes place, and bacteria are usually released from the infection threads into the cytoplasm of the host cell. The thickening of the cytoplasmic lining and the associated rapid multiplication of the bacteria to fill most of the cells takes place to a more limited extent than in the normal nodule, and, as indicated above (p. 388), there is a definite relationship between the size of the region in which this occurs and the strand development. The

bacteria which thus multiply in the enlarged cells exhibit a swelling and a loss of staining power, but it is noticeable that they remain less swollen and more rod-like than do the bacteria in the corresponding tissue of the normal nodules. It would appear, therefore, that the swelling of the bacteria, as well as their rapid multiplication almost to fill the enlarged cells, is closely related to an efficient vascular supply. The meristem at the distal extremity develops normally, at any rate for some time, although the vascular strands have not grown out as far as that region of the nodule.

These nodules with defective vascular strands do not as a rule attain full size, degeneration of the nodule soon taking place (Plate 30, fig. 17). This degeneration first occurs in the swollen cells of the older tissue, but soon extends into the protoplasmic cells of the meristem cap, many of which can be seen to be filled with masses of bacteria. The bacteria attack the contents of the host cell (Plate 30, fig. 18). In a degenerating nodule of this type, the swollen cells may often be seen to contain neither nucleus nor any indication of cytoplasm, but appear to be filled merely with masses of bacteria (Plate 30, fig. 18). The bacteria are usually in the form of short rods or cocci, the "preswarmers" of Bewley and Hutchinson.

The nodules possessing a weak development of vascular strands thus exhibit four points of difference from the normal nodule. First, the region of the "bacteroidal tissue" is limited in proportion to the development of the strands; secondly, the swelling of the bacteria is less marked than in the normal nodule; thirdly, the bacteria soon become actively parasitic and destroy the protoplasm of the host cell, and, fourthly, the meristem cells often become filled with bacteria.

#### H — *Discussion.*

The investigation of the nodules developing on plants grown in the absence of boron thus throws light on several points in connection with the normal functions of the vascular strands in the nodule.

With regard to the growth of the nodule tissue, the first point of interest concerns the relation of the vascular strands to the formation and growth of the meristem cap of the nodule. In nodules having a weak development of strands attaining only one-third or one-half the length of the nodule, the meristem cap at first appears normal and shows active cell division. Thus it can behave in an apparently normal manner although ill supplied with vascular strands and though separated by a considerable distance from the extremity of these. In these abnormal nodules, if the meristem is formed, it is, as a rule, infected during the degeneration of the nodule and many of the protoplasmic cells are

filled with masses of bacteria. In this connection an interesting theory is advanced by Pierce (19), who considers that the freedom from infection of the meristem in normal nodules is due to its rapidity of division, which results in a layer of new cells being continually produced in front of the advancing bacterial threads. In the normal nodules here considered, it seems reasonable to suppose that the deficiency of vascular supply delays the growth of the meristem so that the bacterial strands can reach it. The initial swelling and vacuolation of the cells in the primary nodule and in the region behind the meristem in the secondary nodule growth is apparently independent of the strand development, since it occurs in nodules having no strands and, where weak strands are present, it occurs beyond their extremities.

With regard to the effect of vascular supply on the bacteria within the nodule, it is clear that the branching of the infection thread through the tissues is independent of the presence of strands. The release of bacteria from the infection thread is the next point of interest. This question was discussed by Mazé (17), who thought that there was a causal connection between the development of the vascular strands and the release of bacteria into the cytoplasm of the host. A connection between the presence of vascular strands and the release of bacteria from the threads seems to be indicated by the fact that in nodules having no strands the bacteria are usually embedded in threads and masses of zooglœa. They are not always so embedded, however, even where no vascular strands are formed, and, where short strands are present, the bacteria are released into the cytoplasm in cells lying at a considerable distance beyond their extremities. Thus, while the release of the bacteria into the cytoplasm may be stimulated by the diffusion of substances from the strands, as Mazé supposed, there is no intimate connection between their release and the growth of the strands.

The case is different when we consider the final stage in the development of the infected cells, namely, the vast multiplication of the bacteria in the cytoplasm and their swelling up to form faintly staining sausage-shaped rods and "bacteroids." There is a close connection between this change and the growth of the vascular strands. The formation of the swollen-rod forms and of the typical "bacteroidal" tissue does not occur in the complete absence of vascular strands, and, furthermore, where strands grow into the nodule, the development of this swollen "bacteroidal" tissue is closely proportional to their growth, not extending beyond their extremities (*see* p. 388). Thus not merely the presence, but the proximity of the vascular supply is necessary to the formation of the swollen forms of bacteria typical of the developed nodule.

Equally striking is the change in physiology of the abnormal nodules. As described above, analysis shows that such nodules, on plants grown without boron, were fixing very little nitrogen compared with those on normal plants, where considerable fixation occurred. Thus, in the abnormal nodules, a defective vascular supply is found to be associated with a greatly decreased ability to fix nitrogen.

The development of the vessels in the nodules is thus connected on the one hand with the rapid multiplication and change in morphology of the bacteria in the cytoplasm, and, on the other hand, with the nitrogen metabolism.\* It is reasonable to connect these effects with the supply of carbohydrate material brought into the nodule by the vascular strands. There is also another function of the strands that may, perhaps, be of importance—namely, the efficient removal of the products of metabolism of the bacteria. This function was discussed by Golding (11), who found that in cultures of *Bacillus radicum* greater nitrogen fixation could be obtained where the metabolic products were removed by means of a porous filter. The investigation of nodules on plants grown without boron, while it emphasises the great importance of the vascular supply in the nodule, has not at present enabled us to distinguish between these two functions.

Finally, the present investigation throws an interesting light on the relationship of the bacteria to the host plant. The failure of the vascular supply in the nodule results in the bacteria attacking and destroying the protoplasm of the host. It is maintained by Pierce that in normal nodules the bacteria are parasitic in the sense that they produce degeneration of the nuclei of the infected cells. In our material this degeneration does not occur until very late in the life of the host cell, some time after it has attained its full size and development. The state of affairs in the abnormal nodules, however, shows that under some conditions the bacteria are capable of decomposing the protoplasm of the host cell. Now, in the normal nodule cell very much larger numbers of bacteria are produced by multiplication. Why have these attained such large numbers without destroying the protoplasm of the cell in which they lie? The well-developed vascular strands have in this case introduced a factor that has altered the metabolism of the bacteria so that they do not destroy the protoplasm of the plant—at any rate, till the nodule

\* Nobbé and Hiltner (18) concluded that the bacteria in the nodule that do not reach the "bacteroid" stage are harmful rather than beneficial to the host plant, and that these unchanged bacteria have no connection with nitrogen fixation, which commences with the formation of bacteroids.

decays after its period of functional activity. The nature of this factor seems to be indicated if one considers the change of energy supply in the environment of the bacteria brought about by the vascular strands.

It is well established that the nitrogen metabolism of bacteria can be altered fundamentally by the nature of the energy material available to them. Thus Doryland (7), in studying the production of ammonia from organic nitrogen compounds by soil bacteria, found that this could be reduced or entirely inhibited by the addition of sugar to the medium. Similarly, the work of Berman and Rettger (1) shows that the decomposition of proteins by bacteria in culture is reduced by the addition of carbohydrate. In such examples the lessened decomposition of the organic nitrogen compounds is due to the fact that the carbohydrate added is utilised by the bacteria in preference as a source of energy. In the normal nodule there is a closely analogous state of affairs. The vascular strands supply the bacteria with carbohydrates, which they utilise as a source of energy in preference to the protoplasm of the host cell. In the nodules on plants grown without boron, in which the vascular strands are absent or defective, this supply of carbohydrate is cut off, and the bacteria therefore utilise the host's protoplasm as a source of energy. This may also explain why in the abnormal nodules the bacteria multiply so rapidly in the very protoplasmic cells of the meristem region, as described above. These cells are often found to be filled with masses of bacteria, while the vacuolated cells in the interior of the nodule are comparatively free (Plate 30, fig. 19).

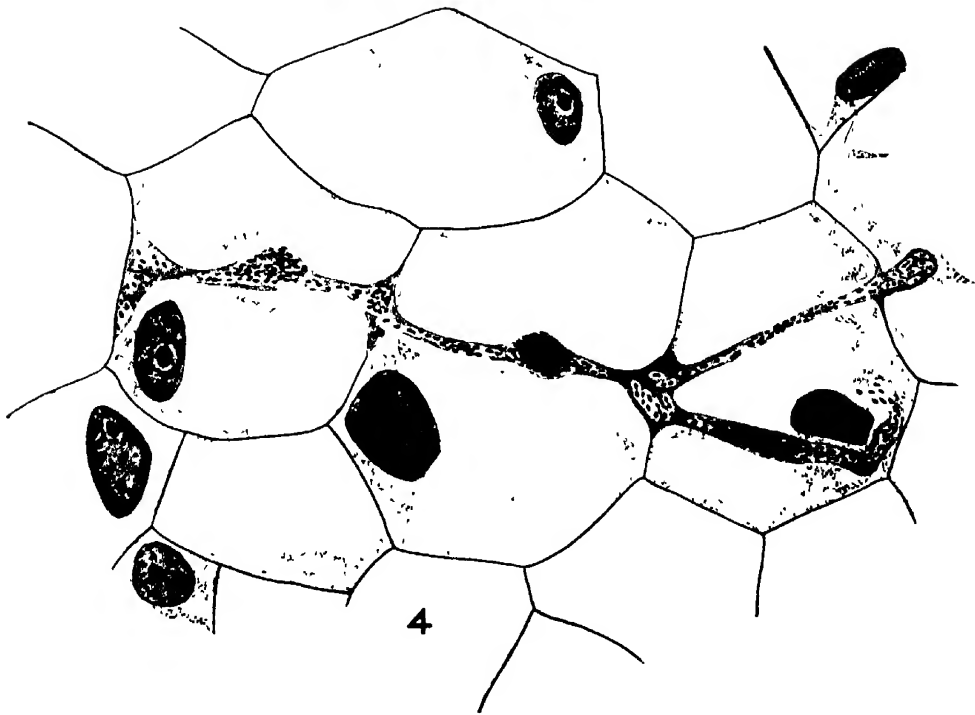
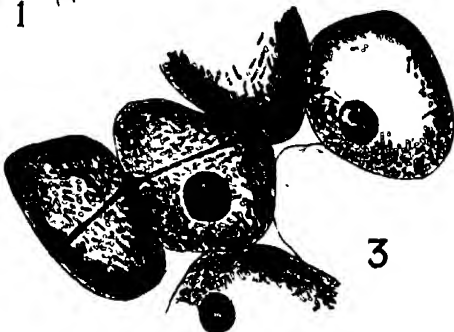
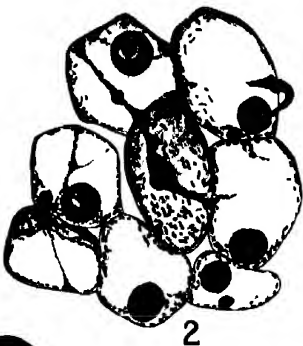
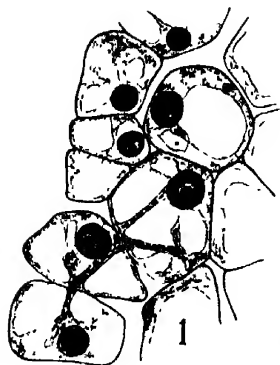
It seems possible that in the nodules having no vascular supply we have reproduced a condition of affairs reminiscent of the primitive relations existing between the nodule organism and its host plant, and that, in the course of evolutionary history, the development of vascular strands running into the nodule has so altered the metabolism of the parasite as to make it harmless and eventually beneficial to its host. Finally, it is suggested that the ultimate degeneration of a normal nodule requires detailed investigations in the light of the above results.

#### I.—Summary.

(1) The work described deals with the growth and functioning of nodules on *Vicia faba*, comparing those grown in culture media from which boron has been excluded with those supplied with boron.

(2) In the absence of boron the vascular supply of the nodule is defective. The strands are often entirely absent, or, where present, are weakly developed, running only a short distance into the nodule.





(3) The nodules having no vascular strands remain minute, and are usually buried in the cortical tissues. In plants grown without boron, the number of nodules that attain macroscopic size is much reduced as compared with normal plants.

(4) In the nodules without vascular strands, the bacteria do not swell out to form the so-called "bacteroids." When weakly developed strands enter the nodule, the amount of tissue containing bacteroids is closely correlated with the extent of the strands.

(5) In the plants bearing these abnormal nodules very little nitrogen is fixed, the quantity fixed per nodule being, in one experiment, less than one-tenth of that fixed in normal plants. The defective vascular supply is thus accompanied, on the one hand, by a reduced development of "bacteroid" forms, and, on the other hand, by reduced nitrogen fixation.

(6) In the absence or weak development of vascular strands in the nodule, the bacteria tend to become parasitic, attacking the protoplasm of the host cell. This attack is chiefly directed towards the more densely protoplasmic cells of the nodule. It is suggested that this change in the relations between the micro-organism and its host is connected with the loss or reduced supply of the carbohydrate energy material normally brought into the nodule by the vascular strands, the bacteria thus being reduced to making use of the protoplasm of the host as a source of energy.

#### DESCRIPTION OF PLATES.

##### PLATE 27.

FIG. 1.—Cells from the young tissue of a nodule, close behind the apical meristem, showing their infection with bacterial zooglœa. In the middle cell, a strand containing a single row of bacterial rods can be seen extending to the nucleus.

FIG. 2.—Cells from slightly older nodule tissue, showing swelling and vacuolation. Many of the bacteria have escaped from the infecting zooglœa and lie free in the cytoplasm. They still consist of thin rods and are not yet swollen into bacteroids.

FIG. 3.—Cells of the bacteroidal tissue from the older parts of the nodule. The cytoplasmic lining of the wall has become thickened and is filled with bacteria in the swollen "bacteroid" condition. Crossing the cells can be seen the remains of the infecting zooglœa still containing bacteria in the thin rod stage.

Figs. 1 to 3 are drawn from a section of a normal nodule grown in the presence of boron.

FIG. 4.—Passage of the infecting thread of bacterial zooglœa through three cells, showing the contained rods surrounded by a clear zone. Cells from a nodule grown without boron.

##### PLATE 28.

FIG. 5.—Proliferation of cells (a) in the cortex of the root to form the primary nodule, (b) portion of stele.



FIG. 6.—Young nodule showing the beginning of secondary lengthening and formation of swollen vacuolated cells in the proximal region. (a) cortex of nodule, (b) meristem, (c) swollen vacuolated cells.

FIG. 7.—Nodule in which bacteroidal tissue has begun to form, showing its close association with a vascular strand. (a) nodule cortex, (b) meristem, (c) swollen vacuolated cells, (d) bacteroidal tissue, (e) vascular strand.

FIG. 8.—Longitudinal section of root and nodule showing a further growth of the bacteroidal tissue associated with an extension of the vascular strands. (a) nodule cortex, (b) meristem, (c) swollen vacuolated cells, (d) bacteroidal tissue, (e) vascular strand, (f) stele.

FIG. 9.—Longitudinal section of a large nodule. The strands extend as far as the meristem cap and the bacteroidal tissue almost fills the interior of the nodule. (a) meristem cap, (b) swollen vacuolated cells, (c) bacteroidal tissue, (d) vascular strands.

#### PLATE 29.

Infection and abnormal nodule development in *Vicia faba* grown in absence of boron.  
Transverse sections of the roots.

FIG. 10.—A portion of the piliferous layer of the cortex showing an infection thread of bacterial zoogloea passing down it.

FIG. 11.—Part of a section showing at (a) an infection thread passing through the cells of the cortex (b).

FIG. 12.—Part of a section, showing the early multiplication of cells in the deeper layer of the cortex to form the young nodule. (a) the root hair, (b) a portion of the infection thread, (c) enlarged cortical cells, (d) normal cells of the cortex, (e) the young nodule.

FIG. 13.—Young nodule developing without the extrusion of vascular strands from the stele. (a) endodermis separating the stele (b) from the nodule (c).

FIG. 14.—A larger nodule without vascular strands, showing the absence of bacteroidal tissue and of definite meristem. (a) a portion of the infection thread entering at the side of the nodule.

FIG. 15.—A nodule without strands, in which the bacteria are attacking the tissues. At (a) masses of bacteria have broken the cells of the host and turgid uninfected cells can be seen projecting into the disintegrating tissue.

#### PLATE 30.

FIG. 16.—Transverse section of root of *Vicia faba* grown without boron showing a nodule having a vascular strand projecting but a short distance into the nodule. (a) degenerating nodule tissue, (b) short vascular strand, (c) cortex, (d) endodermis of nodule, (e) endodermis of stele.

FIG. 17.—Transverse section of a root of *Vicia faba* grown without boron, showing a nodule along which the vascular strands extend half-way. At (a) a vascular strand is cut across near its base. Some bacteroidal tissue has formed at (b), but the bacteria have attacked and destroyed the cell contents. At (c) is the infecting strand of bacterial zoogloea passing through the cortex.

FIG. 18.—A further enlarged portion of a nodule in similar condition to that shown in fig. 18. The degeneration of the bacteroidal tissue can be seen. The infected cells are filled with masses of bacteria (a) that have destroyed the nucleus and cytoplasm. At (b) a branch of the infecting strand still persists.



FIG. 5

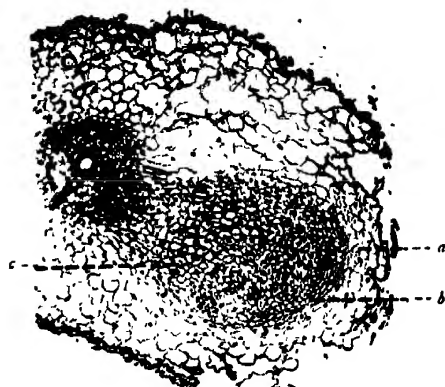


FIG. 6

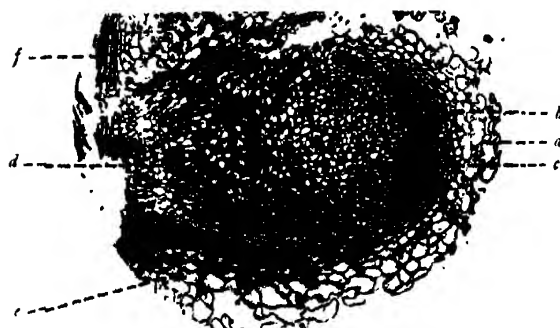


FIG. 8

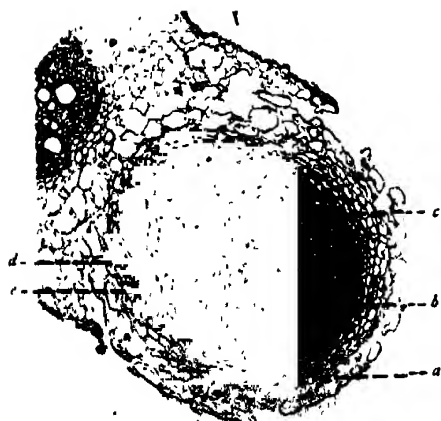


FIG. 7

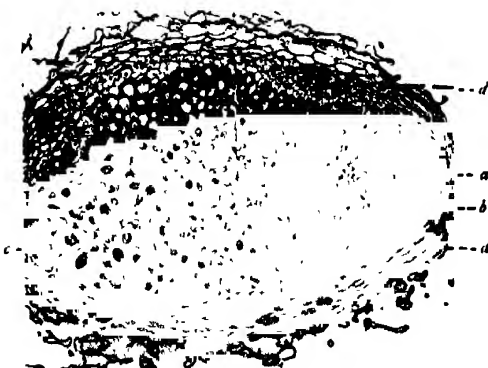


FIG. 9



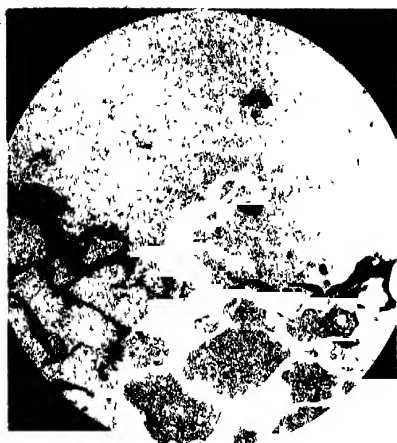


FIG. 10

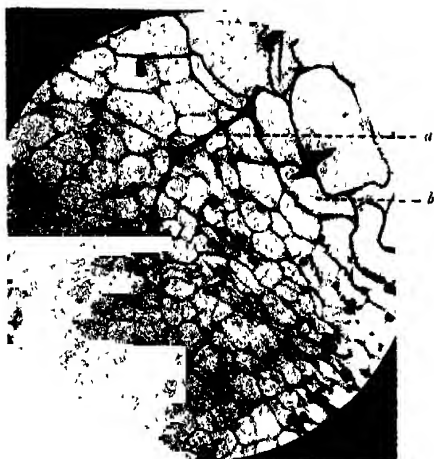


FIG. 11

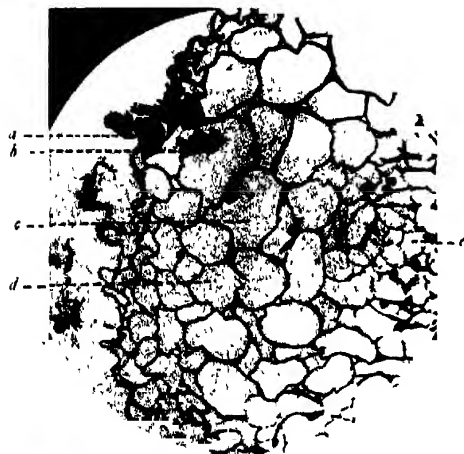


FIG. 12

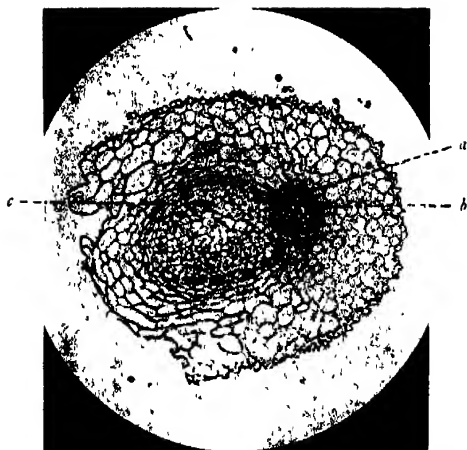


FIG. 13

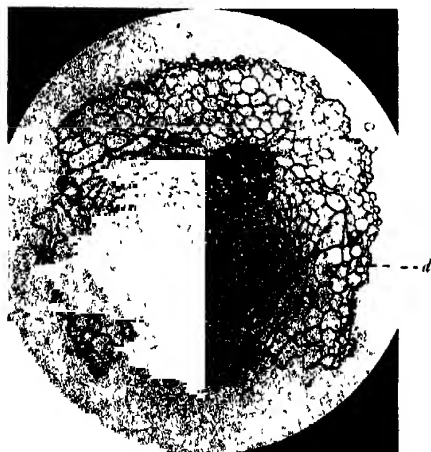


FIG. 14

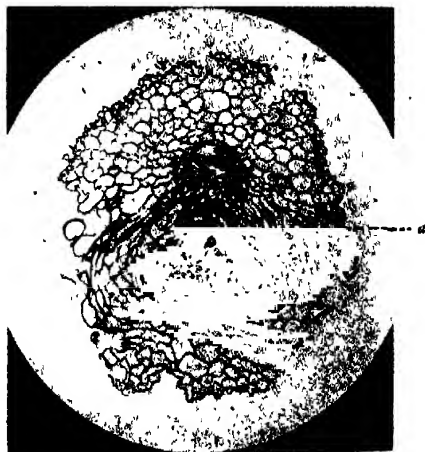


FIG. 15



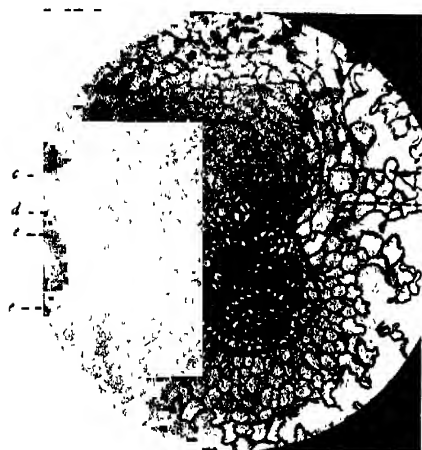


FIG. 16

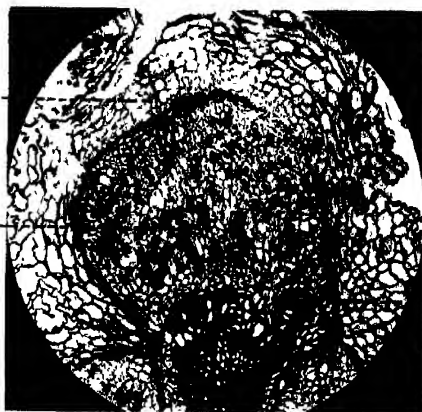


FIG. 17

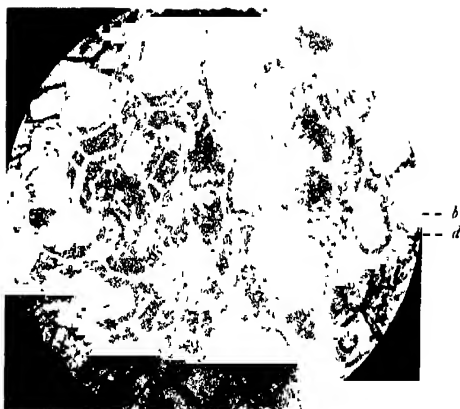


FIG. 18

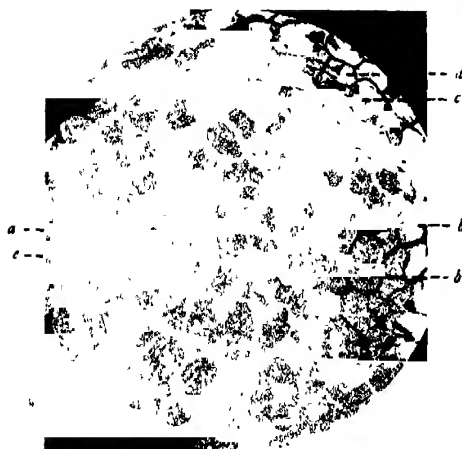


FIG. 19



FIG. 20



FIG. 19.—Section of a nodule grown in the absence of boron and into which no vascular strands have entered. At (a) can be seen an infection thread of bacterial zooglœa that expands at (b) to form lobate processes. At (c) the nodule meristem is seen, the cells being filled with masses of bacteria. (d) cortex of nodule, (e) enlarged cell tissue in the centre of the nodule.

FIG. 20.—Section showing the distal portion of a normal nodule grown in the presence of boron showing at (a) the nodule cortex, at (b) the meristem free from bacterial infection, at (c) the swelling of the cells behind the meristem which are being invaded by the bacterial infection threads visible at (d)

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*The Electrical Characteristics of an Arc Lamp (Direct Current)  
Measured by Biological Effect.*

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(Communicated by Prof. Leonard Hill, F.R.S.—Received April 28, 1925.)

The experiments about to be described are part of a series made with the object of determining the most effective form of arc lamp for curative work, and refer particularly to the measurement of biological effectiveness, and to the variations observed in this quality when the electrical characteristics of the arc are changed. The work was initiated as the outcome of a large amount of experimental work with arc lights, undertaken by Prof. Leonard Hill and his colleagues, during which it became obvious that the biological effectiveness of the arc lamps was varying. To Prof. Hill the writer is indebted for the following Introduction —

“It has been shown that the effective rays on the epidermis are the ultra-violet of wave-length 3100–2500 A.U., and that the visible and dark heat rays by raising the temperature of the epidermis accelerate the action of the ultra-violet rays (Eidinow and Hill).\* The visible rays have, if powerful enough, a heating effect, which may be sufficient to burn and produce the inflammatory effects of heat. Focussing the visible rays on the skin until the maximal endurable temperature of the surface of the skin is reached, it is found that the temperature under the skin may rise even to 47° C. (Sonne, A. C. Campbell and Leonard Hill).†

“The dark heat rays do not heat the subcutaneous tissues so much, but, absorbed more by the surface of the skin, warm that, while the visible rays largely penetrate to and warm the blood beneath the surface.

“Not only the ultra-violet rays, but heat, sufficient to produce erythema acting locally on the skin, can raise the hæmobactericidal power of the blood (Colebrook, Eidinow and Leonard Hill).‡ The ultra-violet rays do not heat the skin sensibly and can produce inflammatory reactions (sunburn) and raise the hæmobactericidal power of the blood, even when acting on a cool skin.

“After a latent period of a few hours these rays produce reactions in the

\* “The Biological Action of Light,” ‘Roy. Soc. Proc.,’ B, vol. 95, p. 163 (1923).

† ‘British Journ. of Experimental Pathology,’ vol. 5, p. 317 (1924).

‡ ‘British Journ. of Experimental Pathology,’ vol. 5, p. 54 (1924).

living cells of the epidermis, resulting in local erythema and cedema, leucocytosis, local and general, and peeling of the skin, followed by pigmentation. Using a blackened thermopyle to measure the energy of the ultra-violet rays of various wave-lengths, and using equal intensities, it has been found that the wave-lengths of about 3000-2900 are most effective on the skin, that is the ultra-violet rays which come through on clear days with high sun. Photography, involving a chemical reaction, reveals the intensity of the various lines in the spectra of arc lamps in the blue-violet and ultra-violet region, but the photographic plate is exceedingly sensitive to, and is acted upon by, the short ultra-violet rays which cannot penetrate the horny layers of the skin.

"The intensity of various lines in photographs of arc spectra cannot therefore be used as a measure of therapeutic effectiveness. An intensive group of rays in the long ultra-violet region may penetrate the epidermis and have no action there, but be absorbed and heat the subcutaneous blood, as visible rays do; while an intense group of rays in the short ultra-violet region may fail to penetrate the horny layers of the epidermis and so have no action on it

"J. E. Barnard has shown that the very short ultra-violet rays cannot penetrate through a single bacterium, one microbe is screened from the lethal effect of these by another lying over it.

"To estimate dosage for patients the period in minutes is determined which produces slight, moderate or severe erythema of the skin, placed at right angles to the arc at a standard distance, but skins vary in thickness, pigmentation, and sensitivity. A dose which just produces erythema in a fair thin skin may have no effect on a brunette's darker, thicker skin."

The method chosen for use in these experiments is the measurement of the time taken by the arc rays to kill small samples of infusoria (*e g*, paramoecia) taken from an old, colourless culture and exposed in a quartz-faced cell at a known distance from the arcs under test. This method has been elaborated by L. Hill and Eidinow from that used in their research on the effects of distance and temperature upon the killing power of ultra-violet light, and the apparatus used here was similar to that described by them.\*

A small shallow cell was used, backed and fronted by thin quartz plates, the organisms being exposed to the light under test shining through the quartz cell, and observed through a microscope of low power.

The temperature of the cell was kept constant by passing a stream of water at room temperature over the back of the cell. Many previous experiments

\* 'Roy. Soc. Proc.,' B, vol. 95, p. 172 (1923-4).

have shown that no significant rise in temperature occurs in a cell so cooled.

A warning must here be given about the character of the culture used. The

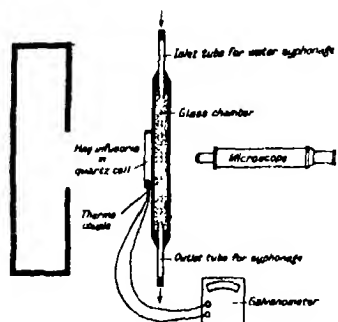


FIG. 1.

culture of infusoria must not be made in an infusion of hay and straw, because the yellow colour, due to a flavone which dissolves out, is highly absorbent of ultra-violet rays, and so protects the infusoria (Eidinow and L. Hill)

Cultures made in dried grass—grass which has been weathered out of doors for a long period—are colourless, and the infusoria in such can be killed in a fairly uniform time by a given dose of ultra-violet radiation. The

same depth of cell must be used and approximately the same number of infusoria in the content. If the infusoria are crowded, or the culture fluid cloudy, the infusoria are more protected. Comparative tests of arcs made on the same day with the same culture are then most satisfactory.

The usual difficulty in determining the "end point" was experienced, there always being a few organisms of greater resistance to ultra-violet radiations than others, these continuing to move after the majority were still. In practice, there appears to be a fairly definite time when there is a sudden decrease, followed soon by a cessation of movement of the majority of organisms, and in these experiments this cessation of movement of the majority was taken as the "end point" as being more easily comparable for short and long killing times.

There is reason to suppose that the photoelectric and sequential chemical changes, which take place in the cells of the epidermis on exposure to ultra-violet light, are similar in kind to those which occur in the infusoria and cause their destruction.

The effect observed in the infusoria is increasing granulation of their protoplasm, just before entire cessation of movement; later on an alteration to a spherical shape takes place, and actual rupture and discharge of granules may occur.

The alterations of the skin shown by erythema, oedema, desquamation and pigmentation, result from injury to the living cells in the deeper layers of the epidermis, and the beneficent effects that follow exposure to ultra-violet light appear to be connected with the reactions which lead to repair and immunity to such injury. Now we know that the shorter the wave-length of an ultra-

violet ray, the less it penetrates the outer horny layer of the skin, also we know that, with a possible reservation for certain wave-lengths, the shorter the ultra-violet ray the stronger the lethal action on living micro-organisms. Herein lies a possible weakness of the infusoria method of measuring the effectiveness of light sources on the skin, because short rays from one source, richer in these than another, may hasten the death of these animalculæ, whose tiny bodies are easily penetrable, but may be insufficiently intense to penetrate the outer layers of human skin enough to make an appreciable difference to the living cells below.

We must remember that artificial sources of ultra-violet light supply us with intense radiations, down to 200 A U. wave-length, much shorter than those which reach the patients in heliotherapeutic establishments, while the shorter sun rays are absorbed by ozone in the upper layers of the atmosphere, the longer ultra-violet rays which reach us from the sun may be of great intensity. With high sun and clear sky rays as short as 2900 reach us, and (as said above) the rays from 3000-2900 are most effective in producing sunburn.

The following experiment shows how the lethal effect on paramœcia may differ from the erythema dose on the skin. Upon substituting a positive carbon carrying a small aluminium metal core for a "white flame" positive carbon previously used, the paramœcia were killed in about  $\frac{1}{2}$  of the time, but upon exposing two small areas of the skin of the abdomen, respectively, to the "white flame" and to the aluminium cored arcs, the degree of erythema produced was almost identical on each.

The intense short rays of the aluminium arc acted on the infusoria, but failed to penetrate the horny layer of the skin. Keeping to the same poles—viz., "white flame" carbons—the paramœcia method, nevertheless, allows us to measure the effectiveness of arc lamps, and the comparative results are generally applicable to the skin. In fact, a long series of observations by A. Eidinow, using "white flame" carbons, show a close relation between infusoria-killing time and the erythema dose for white skins.

#### *Values Measured.*

The tests were made by varying the current passing through the arc, the P D. or voltage between the carbons, and the length of the arc, or distance between their ends. Here it must be noted that a carbon arc is inherently instable, and that it is not possible to make electrical measurements of its performance with the great precision usual in such work.

Mrs. Ayrton\* says "While the external resistance can be kept absolutely constant, the length of the arc cannot, for it is quite impossible, either automatically or by hand, to keep the carbons moving towards each other at exactly the same rate at which they burn away. Consequently what is *called* keeping the length of arc constant is really allowing it to become slightly longer than the desired length, and then bringing the carbons together till it is slightly shorter, so that the curve connecting time and length of arc, when the length of arc was supposed to be constant, would be a zig-zag, not a straight, line.

"Similarly, what is called a constant current is not really constant, for it is impossible to lengthen or shorten the arc, and at the same time alter the resistance by exactly the right amount to keep the current constant. . . Nevertheless, it is possible to keep both the length of arc and current constant within certain limits, which may be made very narrow by careful experimenting."

To control the length of the arc a slight alteration had to be made to the lamp, which was of the automatic type and which consequently was designed to maintain the arc burning steadily at a length suitable to the conditions at any instant, and not to assure a fixed length.

Fig. 2 is a diagrammatic sketch, showing how a Westminster-Eidunow lamp was adjusted to burn with the carbons at a nearly constant distance apart.

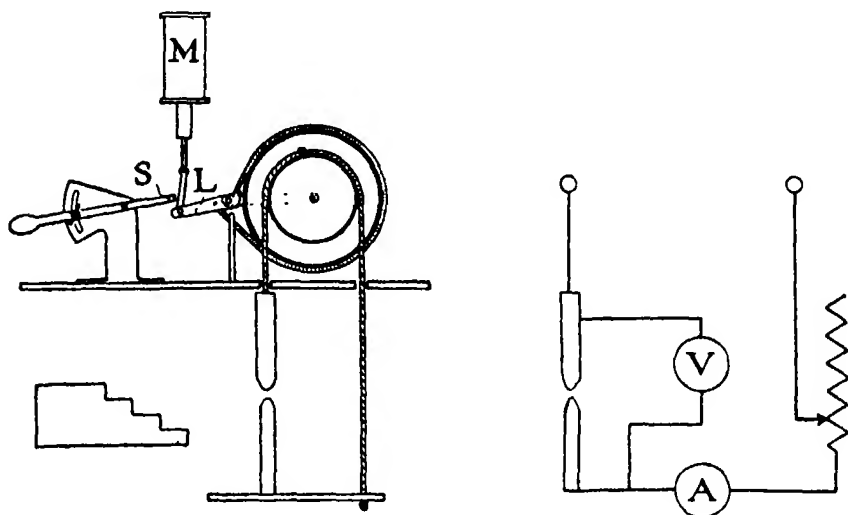


FIG. 2.

The carbons come together by gravity when the current is off, and when the lamp is started the solenoid (M) draws up the lever (L) of the band brake,

\* 'The Electric Arc,' Hertha Ayrton.

which turns the wheel until the carbons are separated so far that the solenoid (M) has its force counterbalanced by that of another shunt solenoid (not shown). By adding the stop (S) on the adjustable lever shown on the left, the travel of the lever (L), and consequently the separation distance of the carbons, can be limited as desired. In this way any burning away of the carbons is made good every time the lamp goes out, and readjusts itself; the carbons have to touch before the brake can get another hold and they are then re-separated to the original distance apart.

In practice the distance was set by using a step gauge, as shown on the left (fig. 2), each step being cut 1 mm. short of the desired length of the arc, so that a reasonably accurate mean gap might be maintained by periodically extinguishing the arc and allowing it to re-establish itself. The connections for volt and ampere meters are also shown, and the measurement of the energy consumed in the arc, within reasonable limits of accuracy, presented no difficulty.

*Varying the Length of the Arc, but keeping the Energy Constant.*

In the first experiment the length of the arc was decreased step by step, and the variable resistance was adjusted to ensure that the energy consumed in the arc itself was as nearly constant as possible.

Infusoria were exposed in a cell, at right angles to the carbons of the vertical arc and three feet distant.

Length of arc Millimetres.	Volts.	Ampères.	Energy Kilowatts.	Killing time. Minutes
50 .	85	20 0	1 70	5
40	71	23 5	1 67	5
30	61	27 5	1 68	5
20	54	30 4	1 64	5
10	50	34 0	1 70	5
5	40	40 0	1 60	5½ (nearly)

In all but the last instance the effect of the arcs on the paramœcia is as nearly as possible the same, and the slight increase in killing time with the 5 mm. arc may well be due to the accidental drop in energy indicated.

It will be observed that the arc was working most efficiently (from an economic point of view) when giving the longest flame. Supposing the supply voltage to have been constant at 150, the voltage across the arc as measured was 85 and so that across the resistance was  $150 - 85 = 65$ . Now the same current

of 20 amperes runs through both arc and resistance, so that the energy used in the arc is  $20 \times 85 = 1.7$  kw. and that consumed in the resistance is  $20 \times 65 = 1.3$  kw. For the short arc, through which the current had to be raised to 40 amperes to give approximately the same energy, the P.D.'s were 40 for the arc and 110 for the resistance, and the energies used were 1.6 in the arc and 4.4 kilowatts in the resistance.

Thus it appears best to use the longest arcs that can practicably be obtained when only a high or moderately high direct current supply is available, and thus to enable the lowest possible resistance to be used, across which a P.D. equal to 20 per cent. of that across the arc itself is maintained. In many cases it will be best to use two or more arcs in series.

There was no difference in the erythema produced by arcs of widely different lengths but of similar energies. To see if the paramœcia and erythema effects agreed, two subjects, G.H. and T.A., each exposed similar parts to the arc, with the help of a suitable screen bound round the leg. First, at 18 inches distance, with 10 mm. arc at 1.8 kw. Secondly, at  $18\frac{1}{2}$  inches distance, with 50 mm. arc at 1.9 kw.

One subject exposed an area for 10, another for 15, and a third area for 20 minutes. The other subject exposed only one patch on each leg to each arc. The erythema was much more marked in the case of G.H. In these two subjects of widely different susceptibility to light, the long and the short arcs of equal power produced the same degrees of erythema.

*Variations in Killing Time with Three Fixed Arcs of Different Lengths.*

In the next experiments the value of the current was varied, the constant lengths of the arcs determining, within a few volts, the values of the P.D.

30 mm. Arc. Infusoria 18 Inches from Arc in Same Quartz Cell.

Volts.	Amperes	Kilowatts.	Killing time.
			Minutes.
62	$14\frac{1}{2}$	0.90	21
64	20	1.28	12
60	26	1.56	11
62	29	1.80	5
62	31	1.92	3
60	33	1.98	3
64	41	2.62	2

Volts.	Amperes.	Kilowatts.	Killing time.
20 mm. Arc.			
53	21	1 10	16½
54	24	1 30	9½
54	29	1 56	7
52	33	1 72	5½
58	31	1 80	5½
56	34 5	1 90	4½
56	37	2 06	3
56	38 5	2 10	2½
60	39	2 35	2½
66	36	2 37	2½
67	38	2 55	2½
10 mm. Arc.			
26	20 }	0 54	95
30	19 }		
39	21 5	0 63	33
27	26 8	0 72	25
34	31 5	1 07	12
39	30	1 17	12½
40	33 5	1 34	9
42	36	1 50	7½
41 5	45	1 86	3½
40	49	1 96	3½
42	52	2 2	2½

The values of energy and killing time for the above three experiments are plotted in fig. 3.

The points for these three arcs lie, with a few exceptions, nearly upon the same regular curve indicated by the thick line, which shows that between these limits the length of the arc, *per se*, has no direct influence upon its power to kill infusoria, but that the killing power of the arc has some regular relation to the electrical energy which it consumes. It is of interest to compare this curve with one drawn with the figures taken from an experiment of Victor Henri.\* The dotted curve shows the relation found by Henri to exist between the energy supplied to a mercury vapour lamp and the time taken to kill *Bacillus coli*; the similarity of the curves is striking.

To return to the arc lamp curve. These points plotted logarithmically

\* 'Comptes Rendus,' vol. 153, p. 265 (1911).



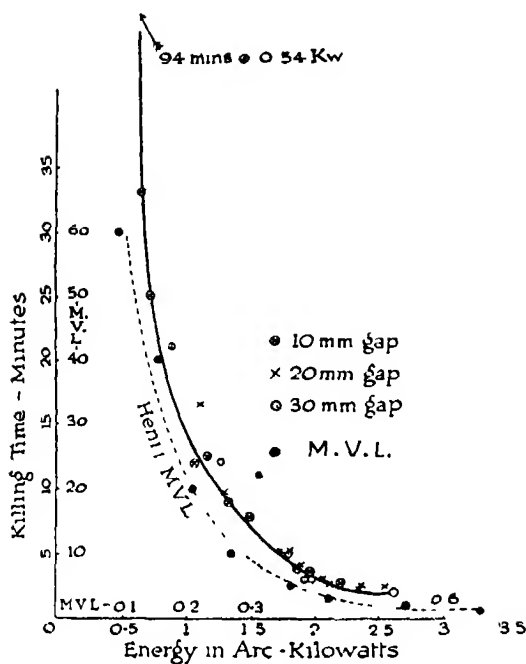


FIG. 3.

fall nearly on a straight line (that corresponding to 94 minutes being no exception), from which the law of the curve was found to be :—

$$TP^{2.5} = K \quad \text{or} \quad T = \frac{K}{p^{2.5}},$$

where

T = killing time in minutes.

P = kilowatts consumed in arc (ignoring those lost in the resistance).

K = a constant depending on the properties of the electrodes ; the distance of cell from arc ; the properties of the infusoria and their containing liquid ; the properties of the quartz cell.

It was hoped to deduce at once from these figures the cheapest way to run this lamp. The cost of producing a given effect is the product of the energy and the time for which this energy is maintained, and it was hoped that by multiplying each applied power in kilowatts by the number of minutes which it took to kill the infusoria, the *kilowatt-minutes* required for a lethal dose thus obtained, plotted against the energy in kilowatts for each dose, would give a regular curve showing a minimum value of kilowatt-minutes, for some critical value of power, or one from which such a minimum might be calculated. No

such simple relation can be found from this test, and the logarithmic values, when plotted, indicated a doubly-curved line with a law of great complexity.

The practical outcome of this test is that arcs formed between these particular electrodes work most economically at from 1.8 to 2.2 kw. consumed in the arc itself; inspection of the curve showing that there is a marked falling-off of effectiveness below 1.8, and but very small increase above 2.2 kw., added to which it was observed that higher powers caused the arc to burn unsteadily and to flare, and in all probability caused the carbons to burn away with undue rapidity.

The rather surprising equality of effects of the long and short arcs of equal powers suggested that the relative effectiveness of zones of equal width in long and short arcs should be compared. We should expect to find that the light from a zone taken in a short arc would have a much greater effectiveness than that from a similar zone in a long arc; the longer arc, to have a total effect equal to that of a shorter one, must have its effectiveness spread out over its larger surface.

Two parallel screens were arranged beside the arc, the first being 67 mm. away from the carbons and the second screen 107 mm. behind the first. The screens contained two horizontal slits 26 mm. in length and 3 mm. wide, and the screens could be together moved up or down before the arc, so that the slits would allow a beam of light approximately parallel and 3 mm. wide to fall upon a small cell containing infusoria and placed just behind the second slit. In this way a series of values could be taken of the killing power of the ultra-violet light from any part of the arc. (Fig. 4 shows the arrangement diagrammatically)

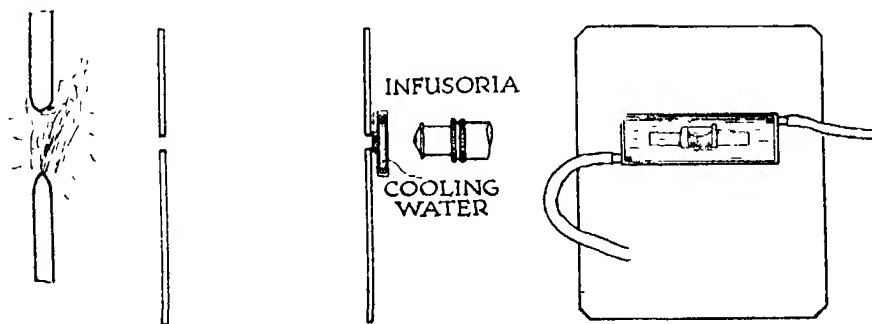


FIG. 4.

Arcs of 41, 22 and 15 mm. were thus explored, beginning with the end of the upper carbon and working downwards, taking a number of zones with their medial planes 5 mm. apart, or in some instances even closer together.

The results are shown graphically by plotting reciprocals of the killing time as horizontal ordinates along a line representing the axis of the carbons; the lengths of the ordinates thus giving a measure of the effectiveness of any particular zone and showing the distribution of biological action.

*Exploration of Arc and Flame.*

*Experiment 1.*—41 mm. Arc. "White flame" carbons as before. Infusoria  
174 mm. from Arc.

Position of Centre Line of Slots.	Volts.	Amps.	K W.	Killing Time of Infusoria	
				Seconds	Reciprocal.
Just above max crater light	—	—	—	225	4 44
Middle of + light band	70	27½	1 93	137	7 40
3 mm. below end of + carbon (Edge of blue flame)	77	27½	2 12	73	13 2
Repeat	75	27½	2 06	75	13 2
5 mm. below (in blue)	74	28	2 07	90	11 1
9 mm. below	74	28	2 07	90	11 1
12 mm. below	68	28	1 90	120	8 33
16 mm. below	75	31	2 32	120	8 33
Repeat	70	29	2 03	90	11 1
22 mm. below	68	24	1 56	135	—
Repeat	75	28	2 10	120	8 33
6 mm. above carbon (bottom of slit)	75	28½	2 10	105	9 53
3 mm. above (just in blue)	75	28	2 10	105	9 53
Bottom of slit in line with end	—	—	—	90	11 1
On carbon inside upper white band	75	27½	2 06	300	3 33

*Experiment 2.—22 mm. Arc.*

Position of Centre Line of Slots.	Volts.	Amps.	K W.	Killing Time of Infusoria.	
				Secs.	Reciprocal
Covering end of + carbon	50	40	2 0	150	165
	55	40	2 2	180	
Just clear of end	54	40	2 16	45	47
				50	
3 mm. down	52	38½	1 95	60	61
	50	39	—	62	
Repeat	59	39½	—	66	58
	53	38½	2 03	53	
	53	39	—	55	66
6 mm. down	48	40	1 92	65	
	48	41	1 97	67	66
	53	38	2 01	65	
11 mm. down	—	—	—	65	63
	57	37½	2 14	57	
	57	37	2 11	68	84
16 mm. down	55	37½	—	91	
	56	37	2 06	80	165
	56	37	—	80	
22 mm. down	56	37½	2 10	170	155
				155	
Top of slot 9 mm. down Central between carbons.	56	37½	2 10	70	14 3
Return to 42 mm. Arc as a check					
Top of slit 19 mm. down	72	28	2 02	140	116
	70	29	2 03	100	
	70	28½	1 99	110	8 62 (Control between carbons)

Fig. 5 is the graph referred to above, showing the above experimental results.

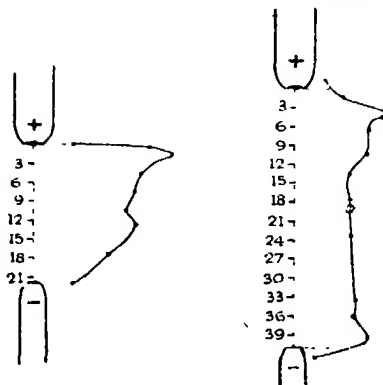


FIG. 5.

Fig. 6 shows graphs for three similar tests made to check the former two; the numerical values of experiments 1 and 2 are comparable with each other, *but not with 3, 4 and 5.*

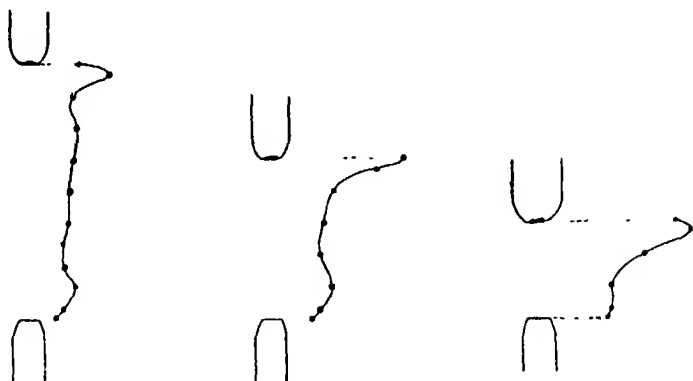


FIG. 6.

Fig. 7 shows a comparison between two more arcs of 15 and 40 mm. and approximately 2 kw. Here the screens were 205 mm. apart, to reduce the

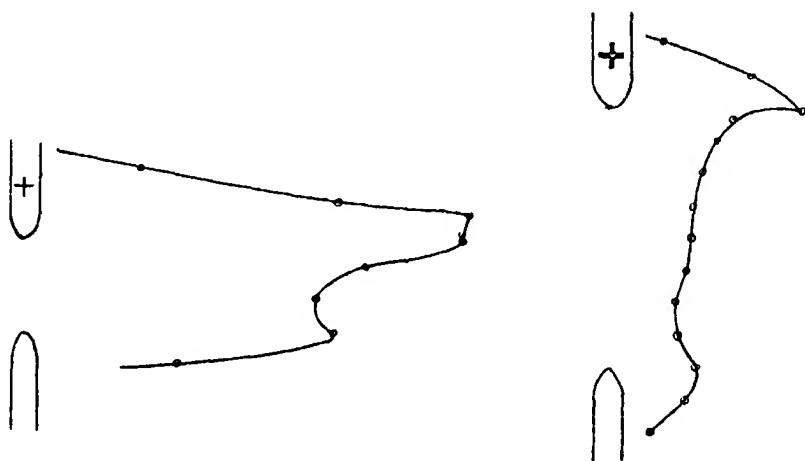


FIG. 7.

killing times more nearly to those in the previous experiments, and the slit in the first screen was lengthened to 215 mm., in case the full width of the flame had not been exposed to the cell in the previous experiments. Readings were also taken above and below the ends of the carbons. The general distribution of energy appears to be much the same as in the previous experiments.

It may, then, be stated that rays from the flame region of an arc may be of

equal or of even greater biological effectiveness than that from the electrodes ; which is contrary to a widely held opinion and quite at variance with previous investigations on the sources of *visible light* from plain carbon arcs (see Mrs. Ayrton).\*

By lengthening an arc we can increase the P.D. across it, which by raising the energy consumed therein increases the effectiveness of the light emitted, and at the same time, by reducing the total current, transfers energy consumption from the resistance to the arc.

Mention may be made of some recent work of Absolon Larsen† on arc lamps as used for therapeutic purposes at the Finsen Institute. Larsen measured the total radiant energy with a bolometer and the photographic effect as indicated by the blackening of photographic paper, the light falling normal to the surfaces and being taken from the positive crater at an angle of  $45^\circ$  with the axis of the carbons, i.e., in the direction of maximum illumination. He gives tables, showing that the radiant heat increases nearly directly as the current, but that the photographic effect increases as some higher power of the current. The attached curves, from Larsen's figures, give for the abscissæ energy consumed in the arc, instead of current as Larsen gives.

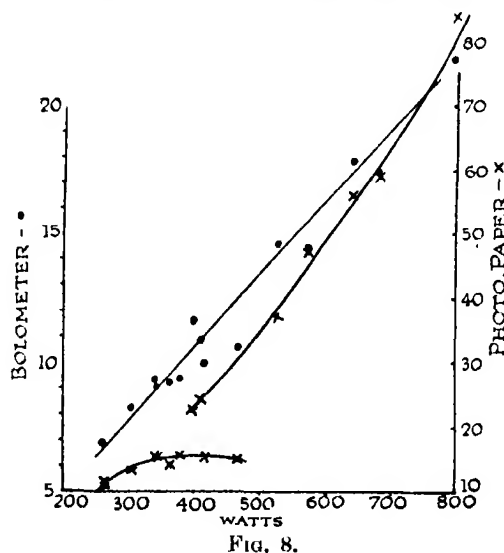


Fig. 8.

Fig. 8 shows the result of an experiment with a hand-regulated low-power lamp. The bolometer points give a fairly regular line, but the lower photo-

\* 'The Electric Arc,' p. 371.

† "Dependence of the Electric Arc Light on Current Strength and Tension" Absolon Larsen, 'Mittelungen aus Finsens Medicinische Lysinstitut.'

graphic readings are very irregular. Fig. 9 shows the curves obtained with a shunt-coil regulated lamp working at higher powers. Both these sets of

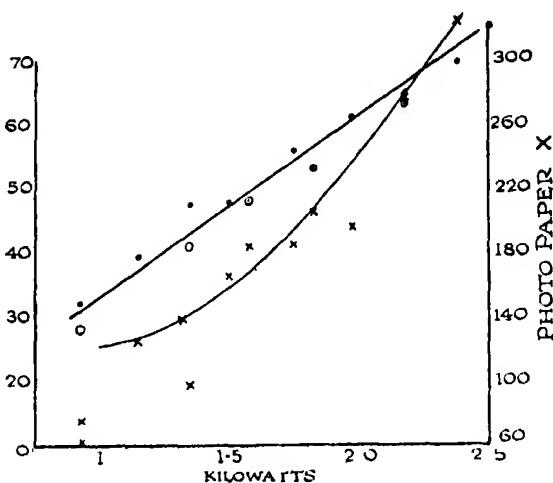


FIG. 9.

curves show that the active rays from the lamps increase in intensity as the energy rises with greater rapidity than does the total radiant energy as measured by a bolometer.

### *Summary.*

The time taken to kill hay infusoria (*i.e.*, paramœcia) is taken as giving a measure of the effect of ultra-violet light on the white human skin, though this must be accepted with some reserve, because these animalculæ can be destroyed by those shorter ultra-violet rays which cannot penetrate the outer horny layers of the skin.

Tests were made of the variation of the killing power on a sample of infusoria, horizontally 3 feet distant from the arc of two arc lamps, using similar carbons and varying --

- Distance between carbons,
- Current flowing,
- Voltage or P.D. between the carbons.

It was found, within the limits of accuracy of the experiment, that the killing power of the light depended upon the electrical energy expended rather than upon any one of these values alone, and that long and short arcs have very nearly similar killing powers, if consuming the same electrical energy.

At 2 kilowatts, the long arc appears slightly to an advantage. The long arc is cheapest to run, less energy being wasted in the resistance. Two or more long arcs run in series would still further lessen the waste of energy in the resistance when using high voltages from the main.

A mathematical law giving the relation of lethal effectiveness to electrical energy was obtained for these carbons, but no definite optimum power for economical working was found. The relation of killing time to energy bears a striking resemblance to that found by Victor Henri for *Bacillus coli* using a mercury vapour lamp.

A test proved that 10 and 50 millimetre arcs consuming the same energy produced the same degrees of erythema on two widely different subjects who were similarly exposed.

The intensity at different parts of the arc was measured by taking the effect of light from 3 mm zones down the length of the arc. The greatest effectiveness was always near the positive carbon, but ultra-violet rays came off from all parts of the region of the flame.

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### *The Effects on Fertility and the Sex-Ratio of Sub-Sterility Exposures to X-Rays.*

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(Communicated by Prof. A. V. Hill, F.R.S.—Received May 2, 1925.)

(From the Department of Physiology, University College, London.)

(PLATE 31.)

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#### *I. Introductory.*

That sterility follows prolonged exposure to X-rays appears to have first been shown in 1903 by Albers-Schonberg (1) who obtained this result by



irradiating rabbits and guinea-pigs. About the same time, Friebe (8) reported atrophy of the testes as the result of exposure. In the following year the histological results of irradiation were the subject of a very careful investigation by Bergonié and Tribondeau (3) on the rat. These authors found that whereas complete degeneration of the germ cells is caused by prolonged exposure to X-rays, the Sertoli cells and the interstitial tissue are not damaged and may even show increased proliferation. These results were confirmed almost immediately by Villemain (23) who also agreed with Bergonié and Tribondeau in finding that resumption of spermatogenic activity may occur where the damage has been insufficient to destroy all the spermatogonia. Simmonds (21) later recorded that some tubules seem to show greater resistance to X-rays destruction. That interruption of the spermatogenic function follows exposure to X-rays was again shown in the rat by Wakelin, Barratt and Arnold (24) and by Regaud (18) on the cat.

Therapeutic and accidental exposure of the human subject also seems to lead to similar results. Philipp (17) records two cases of therapeutic exposures to X-rays which resulted in sterility. Brown and Osgood (5) collected 18 cases of abnormality among X-ray workers, all who had done X-ray work for more than three years showing complete azoospermia.

It is agreed by these authors that no diminution of the sex instincts follows exposure, and this correlates well with the histological findings as regards the interstitial tissue. From this basis the physiological effects of the destruction of the tubules have been studied during more recent years. Tandler and Gross (22) irradiated a roe deer, with the result of degeneration of the tubules. Nevertheless, the irradiated animal cast its antlers and subsequently regrew them in the normal way. The effects on the growth of the accessory organs of reproduction have also been dealt with. Nemenov (13) produced complete atrophy of the germ cells in the dog by repeated exposures without influencing the growth of the prostate. Also, Ancel and Bouin (2) exposed young guinea-pigs, with the results that at 10 months old the tubules were still in an infantile state. In spite of this, however, the sex characters and the seminal vesicles were quite normal.

A further investigation, concerning the breeding performance of the animal after irradiation, and, therefore, of importance from the point of view of the present paper, remains to be considered. Regaud and Dubreuil (19) by means of concurrent exposures and matings studied the onset of sterility in the rabbit. In his first case no live spermatozoa were found in the seminal fluid more than 35 days after the first irradiation, at which time the only impregnation occurred.

After 35 days azoospermia supervened. In the second case live spermatozoa were found for 13 days after irradiation, but no fertilisation was achieved. In each case several does were impregnated with live spermatozoa, and the fact that only one pregnancy occurred leads the authors to suggest that exposure to X-rays may be detrimental to the fertilising powers of the spermatozoa without actually resulting in death or non-production. In any case, it is clear that mature spermatozoa are not necessarily killed outright by such doses as were employed by Regaud and his collaborator (see later).

Exposure of the female results in much the same changes taking place in the ovary as take place in the testis following exposure of the male. The Graafian follicles undergo atrophy, but the interstitial tissue appears to survive. Halberstadter (9) on rabbits, Bergonié, Tribondeau and Recamier (4) on rabbits, Krause and Ziegler (11) on mice and Reifferscheid (20) on mice, dogs and monkeys have all arrived at this conclusion. Since, however, the present paper is not concerned with the ovary, further detail need not be entered into.

The recorded work on the results of exposure to X-rays seems to have been largely initiated by two considerations, first, the histological aspects of the testis degeneration caused by exposure, and second, the effect on the development of the secondary sexual characters and the accessory organs of reproduction produced by the obliteration of the spermatogenic portion of the testis. The later line of work has added strong evidence in favour of the view that the hormone produced by the gonad and directing secondary differentiation is produced by some portion of the gonad other than the spermatogenic one, probably by the interstitial tissue. It is clear, however, that a third line of investigation is possible, *i.e.*, to consider the effects on breeding of exposure to sub-sterility doses of X-rays. Little (12) has dealt with one side of this problem, namely, the condition of the young produced by animals subjected to small doses. This author found that such young were smaller and that many possessed gross abnormalities.

## II. *Methods.*

The object of the experiments described in this paper was to study the breeding performance of mice exposed to a dosage of X-rays insufficient to produce sterility. Since the main object in view was the determination of any effects on the sex-ratio, and since it is impossible to study accurately the effect on the sex-ratio of any treatment when both sexes are abnormal, it was clear that one sex only should be irradiated, and that the other should be kept in a per-

fectly normal manner. Since there is every reason to suppose that in mammals sex is determined by the male by virtue of dimorphic spermatozoa, any attempt at alteration of the sex-ratio should be made on the male, and in the experiments described in this paper therefore the females used were perfectly normal, while the males were irradiated. This procedure eliminated as far as possible disturbing factors, such as pre-natal mortality, introduced by the female.

The exposures recorded by previous workers as sufficient to produce sterility were only a rough guide as to the dosage required for the present purpose. Differences of apparatus and consequent difference in the type of rays produced, together with difference in the distance from the anticathode, no doubt explain the discrepancies in the times of exposures given by various authors. In the present investigation, Coolidge standard tubes were used without a filter. The wave-length was  $0.207 \text{ \AA}$ , giving a soft medium ray. The current through the tube was about 55-60 k.v. and 5 m.a., giving a spark gap (point-point) of 8.3 cms. With the particular apparatus used the nearest to the anticathode at which the animals could be exposed was 18 in., and this was the distance adopted. The animals were protected from general exposure by being put in a hollow lead prism, a hole being made in the bottom so that the scrotum was exposed.

Under such conditions it was found that three exposures of ten minutes each were required to produce the characteristic changes leading to sterility. The dosage preceding the experimental matings was, therefore, limited to one exposure of 10 minutes, giving a dose of 3,000 milliamperere seconds at a distance of 18 in. This dosage did not produce sterility, as the animals were fertile for some months, and examination on killing showed active spermatozoa in the epididymis.

After exposure the animals were mated immediately with one or more does which were segregated as soon as pregnancy was established. The date of birth of the litters was accurately noted and 20 days subtracted to arrive at the date of conception. In order that the sex could be established beyond doubt the young were killed immediately, fixed and dissected. In many cases it is possible to ascertain the sex of young mice at birth by external features; the size, shape and position of the genital tubercle and the presence or absence of rudimentary mammae; in some cases, however, the two sexes approximate so closely that dissection has to be resorted to. To avoid possibility of mistake, fixation and dissection were made a routine.

The irradiated bucks were mostly killed about two to three months after exposure. In each case the epididymis was examined for active spermatozoa by teasing out into Ringer-Locke solution at  $38^{\circ} \text{C}$ ., and examining micro-

scopically the suspension obtained. The testes were cut into fragments and fixed in Carnoy's fluid.\* The sections were cut at 10  $\mu$ .–12  $\mu$ . and stained with iron hæmatoxylin.

### III. *Histology.*

The full sterilisation dosage produced in the testes similar changes to those which have already been described by previous authors, and which therefore need not be further mentioned here.

The changes following the sub-sterility dose are, however, rather remarkable in that a vast number of spermatocytes are present in the tubules, as well as abnormally large numbers of spermatids. Serial sections of nine testes from different animals were examined, and in all this material very few normal tubules were found. In the normal mouse testis tubule there are rarely more than three or four (very occasionally five or six) rows of spermatocytes, and the individual spermatocytes are not unreasonably crowded. (Plate 31, fig. 1.) In the irradiated testes, however, a good many tubules possessed serried rows of spermatocytes packed closely together, and the majority of the tubules possessed a larger number more than usual. In fig. 2A is shown part of a testis section from an irradiated animal, and in fig. 2B is shown a higher magnification of part of the same area. Fig. 3 shows the same type of change in another testis. In one or two tubules evidence of the beginning of necrotic change was observed. Two such tubules are shown in fig. 4. The meaning of this extra number of spermatocytes is obscure, especially as the number of spermatogonia does not seem to be greatly increased. It may, however, be that the increased activity is more apparent than real. If, for instance, irradiation had caused a considerable number of spermatocytes to lose their capacity for mitosis, without actually causing them to disintegrate, the congestion of the tubules would be partially explained.

### IV. *Sex-Ratio.*†

The total number of young produced by irradiated bucks was 493, of which 245 were male and 248 were female. This gives a male percentage of  $49.7 \pm 1.52$ . The normal matings carried out under identical conditions produced

\* Glacial acetic, 1; chloroform, 3; absolute alcohol, 6.

† Calculated throughout this paper as percentage of males, the probable error of which can be calculated from the formula  $0.6745 \sqrt{mf/n}$ , where  $m$  and  $f$  are the proportions of males and females respectively and  $n$  the number of cases. The probable error of the difference between two ratios is calculated from the formula  $\sqrt{(A^2 + B^2)}$ , where  $A$  and  $B$  are the probable errors of the two ratios to be compared.

735 births,\* of which 379 were male and 356 were female, giving a male percentage of  $51.6 \pm 1.24$ . The difference between these two ratios is only 1.9, and since the probable error is 1.95, it is clearly not significant.

These experiments were, however, made in two series. The initial series produced 215 young, of which 110 were males and 105 females. This gives a ratio of  $51.2 \pm 2.29$  which again is clearly not significantly different as it stands from the normal matings. Careful records were, however, kept of the dates of the birth of the litters, and by calculating back 20 days for the period of gestation the date of fertilisation by the buck could be arrived at, and the records were then examined from this point of view. In the first five columns of Table I the births from normal does by irradiated bucks are classified according to the number of days after irradiation that conception took place.

Table I.—Sex-ratio according to Time of Mating after Irradiation.  
(1st Series)

Time of conception. (Days after irradiation.)	No of litters	No. of young.	Males	Fe- males	Totals	Males.	Fe- males.	Per cent Males	Probable Error.
0	3	16	7	9	68	41	27	60.3	$\pm 4.01$
1	2	12	8	4					
2	1	5	3	2					
3	5	35	23	12					
5	1	5	0	5	48	15	33	31.3	$\pm 4.50$
6	2	13	6	7					
7	1	8	3	5					
9	1	6	2	4					
10	2	16	4	12	99	54	45	54.6	$\pm 3.37$
22	1	5	3	2					
23	3	29	18	11					
26	1	7	2	5					
27	1	10	3	7	99	54	45	54.6	$\pm 3.37$
28	1	2	2	0					
29	1	9	5	4					
32	1	6	4	2					
39	1	1	1	0	99	54	45	54.6	$\pm 3.37$
46	1	6	3	3					
48	1	6	2	4					
51	1	6	4	2					
53	1	4	2	2	99	54	45	54.6	$\pm 3.37$
56	1	8	5	3					
Total	33	215	110	105	215	110	105	51.2	$\pm 2.29$

\* The 1,031 normal births dealt with in a previous paper (Parkes, 15) were produced when the colony was in a different laboratory, where conditions were different, and are not strictly comparable with the experimental results discussed in the present paper.

A glance at the first five columns of this table suggests the following tentative conclusions :—

- (a) That during the first few days after irradiation the bucks produce a large excess of males. As a matter of fact, although not shown in the Table, only 2 out of the 11 litters conceived during the first three days after irradiation of the buck produced any excess of females.
- (b) That during the next week, however, females seem to preponderate strongly. Actually, during the next week 7 litters were produced, not one of which had an excess of males.
- (c) That the remaining births conceived 22–56 days after irradiation seem to show nothing very striking as regards the proportions of the sexes.

These results may be strikingly emphasised by grouping the daily totals of conceptions in the following classes.—0–4 days, 5–18 days and 19+ days after irradiation. The totals for the groups are given in the 6th, 7th and 8th columns of Table I, and the male percentage with the probable errors in the 9th and 10th. For the first 4 days after irradiation the conceptions show  $60.3 \pm 4.01$  per cent. of males, whereas the 5–18 days after irradiations group gives only  $31.3 \pm 4.50$  per cent. of males. Subsequently the male percentage rises to the not very abnormal figure of 54.6. The relations of each of these percentages to the normal one of 51.6 and the probable errors of the differences are given in the following table (Table II).

Table II.

Group.	Difference of percentage from normal.	Probable error of difference.	$\frac{\text{Difference}}{\text{Error}}$ .
0–4 days	+ 8.7	4.19	2.06
5–18 "	– 20.3	4.66	4.46
19+ "	+ 3.0	3.59	0.84
Total	– 0.4	2.60	0.15

The percentages for the first two groups are, therefore, significant, and it is therefore to be concluded that following irradiation by X-rays the male percentage shows first a rise and then a fall.

The difference between the male percentages of the first two groups is 29.0, and its probable error is  $\pm 6.02$ . The difference is thus 4.8 times its probable error, and is, therefore, strongly significant.

In view of the striking nature of these results it was decided to repeat the whole experiment again under the same condition and with the same methods. This second series of experiments produced 278 young, of which 135 were male and 143 were female, a male percentage of  $48.6 \pm 2.02$  which is not very different from the normal. The details of the births in this second series are given in Table III.

Table III.—Sex-ratio according to Time of Mating after Irradiation. (2nd Series.)

Time of conception. (Days after irradiation.)	No. of litters.	No of young	Males.	Fe-males.	Totals.	Males.	Fe-males.	Per cent. Males.	Probable Error.
0	3	14	10	4	65	38	27	58.5	$\pm 4.12$
1	1	3	2	1					
2	4	17	7	10					
3	2	13	9	4					
4	3	18	10	8					
5	5	26	11	15	95	33	62	34.7	$\pm 3.28$
6	3	18	7	11					
8	1	3	1	2					
9	2	9	5	4					
11	2	18	5	13					
14	2	12	2	10	118	64	54	54.2	$\pm 3.09$
15	1	5	0	5					
18	1	4	2	2					
19	1	6	3	3					
22	1	8	2	6					
23	4	30	16	14	118	64	54	54.2	$\pm 3.09$
25	2	11	5	6					
27	1	5	3	2					
28	2	14	9	5					
32	1	8	5	3					
34	2	13	7	6	118	64	54	54.2	$\pm 3.09$
35	1	6	3	3					
51	1	4	2	2					
55	1	5	4	1					
57	2	8	5	3					
Total	49	278	135	143	278	135	143	48.6	$\pm 2.02$

A consideration of the first columns of this table shows that much the same results were produced as in the first series. During the first four days after irradiation males are produced in excess. Of the 13 litters produced during this period only 4 showed any excess of females. During the next fortnight females preponderate, the 16 litters of this period including only 3 containing an excess of males. As in the first series, the numbers of the two sexes even up subsequently to the first  $2\frac{1}{2}$  weeks after irradiation.

In the last five columns of Table III the litters of the second series are grouped

as were those of the first. The results are as follows:—the 0–4 days after irradiation the group gives a male percentage of  $58.5 \pm 4.12$ ; the 5–18 days group one of  $34.7 \pm 3.28$ ; while the births after this period gave a percentage of males of  $54.2 \pm 3.09$ .

The relations of these figures to the normal, and the probable errors of the difference are given below in Table IV.

Table IV.

Group.	Difference of percentage from normal.	Probable error of difference.	<u>Difference</u> <u>Error.</u>
0–4 days	+ 6.9	4 30	1.60
5–18 „	– 16.9	3 50	4.83
19 + „	+ 2.6	3.32	0.78
Total	– 3.0	2.36	1.27

As in the first series, therefore, the male percentages for the 0–4 days group and the 5–18 days group are significantly different from the normal, and, again as in the first series, the initial excess of males suddenly swings over to an excess of females. In addition the final litters have again a male percentage not significantly different from the normal. In the case of this second series the male percentage for the total young born is barely significantly different from the normal. This, however, is without meaning, because, owing to the variation in the different time classes, the sex-ratio of the total depends solely upon the relative numbers produced in each of the time classes. The low male percentage of this second series is accounted for by the large numbers of litters produced in the 5–18 days class, where the percentage of females is high. Comparing the first two groups with each other, we find that the difference is 23.8. The probable error of this difference is

$$\sqrt{(4.12^2 + 3.28^2)} = 5.25.$$

The difference is thus 4.5 times its probable error, and is, therefore, strongly significant.

There is an obvious similarity between the results given by the two series of experiments. The two are compared in the following table:—



Table V.—Comparison of First and Second Series.

Group.	First Series.	Second Series.	Difference.	Errors of Difference.	Difference. Error.
0-4 days	60.3	58.5	1.8	5.74	0.31
5-18 „	31.3	34.7	3.4	5.56	0.61
19 + „	54.6	54.2	0.4	4.57	0.085

It is clear, therefore, that there is no significant difference between the two series of experiments, and clear also that the second series entirely bears out the results obtained with the first. Since such close similarity exists between the two series of experiments, and especially since both were performed under identical conditions, it is clearly legitimate to collect the births together to arrive at the results of the experiment as a whole.

Table VI shows the final results obtained in this way.

Table VI.—Aggregate of First and Second Series.

Time of conception (Days after irradiation)	No. of Litters.	No of Young	Males.	Females
0	6	30	17	13
1	3	15	10	5
2	5	22	10	12
3	7	48	32	16
4	3	18	10	8
5	6	31	11	20
6	5	31	13	18
7	1	8	3	5
8	1	3	1	2
9	3	15	7	8
10	2	16	4	12
11	2	18	5	13
14	2	12	2	10
15	1	5	0	5
18	1	4	2	2
19	1	6	3	3
22	2	13	5	8
23	7	59	34	25
25	2	11	5	6
26	1	7	2	5
27	2	15	6	9
28	3	16	11	5
29	1	9	5	4
32	2	14	9	5
34	2	13	7	6
35	1	6	3	3
39	1	1	1	0
46	1	6	3	3
48	1	6	2	4
51	2	10	6	4
53	1	4	2	2
55	1	5	4	1
56	1	8	5	3
57	2	8	5	3
Total . . . . .	82	493	245	248

Grouping this table in the same way as the first two, the following results are obtained :—

Table VII.—Grouping of Table VI.

Time of conception. (Days after irradiation.)	No. of Young.	No. of Males.	No. of Females.	Per cent. Males.	Probable Error.
0-4 days	133	79	54	59.4	± 2.87
5-18 „	143	48	95	33.6	± 2.66
19 + „	217	118	99	54.4	± 2.27
Total	493	245	248	49.7	± 1.52

The relations of these percentages to the normal are shown below :—

Table VIII.

Group.	Difference from normal.	Probable error of difference.	$\frac{\text{Difference}}{\text{Error}}$
0-4 days	+ 7.8	3.12	2.5
5-18 „	- 18.0	2.93	6.1
19 + „	+ 2.8	1.96	1.4

The difference between the ratios for the first two groups is 25.8, and the probable error of this difference is  $\sqrt{(2.87^2 + 2.66^2)} = 3.91$ . The difference is thus 6.6 times its probable error, and is markedly significant.

The combined table, therefore, merely serves to emphasise the conclusions which could be drawn from either series of experiments :—

- (1) That for the first few days after irradiation the offspring of bucks exposed to X-rays show a large excess of *males*.
- (2) That during the next fortnight a still larger excess of *females* appears.
- (3) That following this period the sex-ratio returns to a figure approaching normal.

Possible explanations of these results will be considered later (Section VI).

#### IV. *Fertility.*

The 493 animals born in these experiments were produced in 82 litters, and the average size of litter is thus 6.1. The 735 normal young, on the other hand, were from 116 litters, which thus had an average size of 6.34. The frequency distribution of the normal young works out as follows :—

Table IX.—Frequency Distribution of Normal Litters.

Size of Litter.	No. of Litters.	No. of Young.
1	1	1
2	4	8
3	5	15
4	12	48
5	20	100
6	19	114
7	19	133
8	17	136
9	12	108
10	5	50
11	2	22
Total	116	735

For this series  $\delta^2 = 4.43$  and  $\delta = 2.10$ .\*

The frequency distribution for litter size of the litter from irradiated bucks works out as follows :—

Table X.—Frequency Distribution of Experimental Litters.

Size of Litter	No. of Litters	No. of Young
1	1	1
2	5	10
3	3	9
4	10	40
5	12	60
6	24	144
7	9	63
8	7	56
9	4	36
10	3	30
11	4	44
Total	82	493

For this series  $\delta^2 = 4.94$  and  $\delta = 2.22$ . The standard deviation for litter size is therefore slightly greater in the case of the experimental litters, but not sufficiently so to be of any great significance.

\* This standard deviation of litter size is less than that recorded for normal mice in a previous paper (16). As mentioned in that paper, however, the mice used in the previous calculations had been kept in two colonies. The first, at the Zoological Dept., University of Manchester, in a practically atmospheric temperature, and the second at University College, London, in a temperature artificially maintained at about 65° F. The animals used for the present calculations include only the latter, kept under identical conditions with the experimental animals.

The size of litter may now be considered from the point of view of time of conception after irradiation. From Table VI the following data may be drawn :—

Table XI.—Fertility according to Time of Conception.

Time of conception (Days after irradiation )	No of Litters	No. of Young.	Average size of Litter.
0-4 days	24	133	5.54
5-18 „	24	143	5.96
19 + „	34	217	6.39
Total .	82	493	6.01

This table suggests that the size of litter increases as the time after irradiation increases, but since the male probably plays but little part in determining the size of litter, this result must be accepted with caution.

#### VI.—Discussion.

Since the female controls fertility to the greater extent, and since the females in the experiments described above were perfectly normal, it is not surprising to find that the size of litter shows no very striking features. The general rule that if a male is capable of fertilizing ova at all he is capable of fertilizing as many as can be developed by the female clearly applies here.

The apparent changes in the sex-ratio, however, are striking and require discussion. In the first place, it must be considered whether or not the apparent effect is a true result, a chance coincidence, or an artifact. Let us deal with the second possibility. The sex-ratio for the whole of the experimental young is not significantly different from the normal ; but if this aggregate consisted of two groups, one with an excess of male and one with an excess of female, this result would depend entirely on the numbers of young produced in each class being approximately equal. This aggregate equality is, therefore, no evidence in either direction. In the first series of experiments two of the three classes into which the young appear to fall as regards sex-ratio have abnormal proportions of the sexes, the difference from the normal being in one case 2.08 times its probable error, and in the other case 4.46. In other words, it is 11 to 1 against the first of these differences being due to chance and 1,000 to 1 in the second case. The difference in the case of the second class is thus strongly significant, and suggestive of a true disturbance of the sex-ratio.

The second series of experiments gave exactly the same general results. The 0-4 days after irradiation class had a difference in sex-ratio from the normal

1.6 times its probable error, that is to say, a probability of 6 to 1 against it being due to chance. In the first series of experiments the probability against was 11 to 1. Thus for chance to account for the results as regards the 0-4 days after irradiation class, probabilities of 11 to 1 against and 6 to 1 against would have to have materialised in sequence. On the combined figures the difference from normal is 2.5 times its probable error, a probability of 20 to 1 against a chance result. In the second series of experiments the 5-18 days after irradiation class had a percentage of males different from the normal by 16.9, this difference being 4.83 times its probable error. This means a probability of about 1,800 to 1 against it being a chance result. Thus, for chance to account for the results for this class in both series probabilities against of 1,000 to 1 and 1,800 to 1 would have to have materialised consecutively. On the combined figures the probability against a chance result is about 5,000 to 1.

It is thus unlikely in the extreme that the observed results as regards these two classes in the two series of experiments are due to the chances of random sampling, and some other explanation must therefore be sought of the remarkable fluctuation in the proportions of the sexes, and we are therefore faced with the problem of explaining why radiation of the buck should cause births resulting from conceptions in the first four days after irradiation to have a large excess of male, and births resulting from conceptions 5-18 days after irradiation to have a large excess of females.

In considering variation in the proportions of the sexes it is essential to use as a basis the two factors which govern the proportions of the sexes at birth, *i.e.* :—

(1) The proportions of the sexes at conception.

(2) The amount and sex incidence of pre-natal mortality.

Since pre-natal mortality in the mouse falls preponderatingly on the males it might be possible to explain the low percentage of males in the second class by postulating an enormous amount of pre-natal mortality. This, however, is exceedingly improbable for two reasons :—(a) because it is very difficult to see how it could arise,\* and (b) the not very abnormal litter size found in this class. Any abnormally large foetal elimination must have resulted in the litter size being much less than normal. As regards the first class (0-4 days after irradiation) it is quite impossible to account for the high ratio by assuming

\* It should, however, be noted that on occasion lack of vigour on the part of the spermatozoa appears to increase the amount of pre-natal mortality (Hammond, 10); Davenport, 7, also uses this concept to account for the influence of the male in cases of an hereditary tendency to produce twins.

an increase in pre-natal mortality, and only possible to account for it on the basis of a decrease by assuming that the normal ratio at conception is not less than the very high figure found in this group. Since the size of litter in this group is not greater than normal, as it would be with a much decreased pre-natal mortality, it is clear that for neither group can pre-natal mortality supply the explanation of the anomalous ratios. We are thus reduced to the inference that exposure of the buck to X-rays affects the sex-ratio at conception.

It has been shown elsewhere (Parkes, 15) that there normally exists an excess of males at conception in the mouse, and that this excess is probably due to a differential survival\* rate between the male-producing and female-producing spermatozoa. The only alternative would be to assume a differential power of penetrating the ovum, and this is less probable than the first hypothesis. As regards the results under discussion it is extremely difficult to see how any extension of the less probable hypothesis of the normal excess of males at conception could account for the observed alterations due to irradiation. We may start then with the premises that the disturbance of the original equality between the two types of spermatozoa which normally takes place is in some way aggravated by irradiation, and that during the first four days after irradiation the proportion of male-producing spermatozoa is accentuated, while during the next fortnight the proportion of female producing is increased. It is impossible to avoid the conclusion that the initial increase of males is in some way due to influence exerted on the spermatozoa which are already mature at the time of irradiation, and that the later period of female excess originates in the effect of irradiation on the spermatids and spermatocytes, but even so it is extremely difficult to formulate a hypothesis explaining why the results of irradiation should be reversed in the two periods. It is, in fact, possible to do little more than discuss certain possibilities and those relating to the initial excess of males may be considered first.

(1) Bergonié and Tribondeau postulated the empirical law that : Immature cells and cells in an active state of division are more sensitive to the X-rays than are cells which have already acquired their fixed adult morphological or physiological characters. From this it might be expected that the cells undergoing mitosis in the tubules of the testes would be far more susceptible to irradiation than would the spermatozoa in the epididymis. In this connection it should be remembered that the most striking alteration of the sex-ratio produced was in the period 5-18 days after irradiation, in the young produced,

\* The word "survival" is here used in its widest sense to mean arrival in the Fallopian tubes.

no doubt, from male elements irradiated during the reduction divisions, and that the early alteration amongst the young produced from spermatozoa mature at the time of irradiation was not so marked. If no alteration had occurred during the first few days, the results would have been much more intelligible, and this consideration leads us to consider carefully whether the early variation is really significant. Three facts, however, which have been mentioned before, but which may be emphasised here, suggest that so far as the present data go, the alteration must be regarded as a true result :—

- (a) The correspondence between the two series of experiments is phenomenal if both are chance results.
- (b) Of the 24 litters conceived within four days of irradiation only six had an excess of females, while 14 had an excess of males. In neither series of experiments is the observed result brought about by a big excess of males in one or two litters. The effect is reasonably distributed.
- (c) On the total litters for the first four days the difference of the male percentage from the normal is 2.5 times its probable error.

It may, I think, be taken, therefore, that an actual rise in the proportion of males does take place during the first four days of conception.

(2) Crowther (6) has formulated the hypothesis that the cessation of mitosis which follows prolonged exposure to X-rays results from ionisation of the centrosome, and that the decrease in the number of mitoses follows a probability law according to the duration of the exposure. If Crowther's suggestion is correct certain spermatozoon centrosomes would be ionised as the result of an exposure, but as no question of mitosis is involved in the life of a mature spermatozoon degenerative changes might not immediately ensue. It is, in fact, possible that spermatozoa that had had their centrosome "hit" might fulfil their function of fertilisation. But a further factor is introduced here. The fertilised ovum apparently depends for its first division upon the centrosome introduced by the spermatozoon, and thus an ovum fertilised by an affected spermatozoon would presumably fail to develop. Since, however, the membrane would have been formed, fertilisation by another spermatozoon would be impossible, and the ovum would presumably disintegrate. The result of this would be that the size of litter would be reduced in proportion to the number of "damaged" spermatozoa\* (assuming, of course, that such spermatozoa were

\* Such a case would clearly be an exception to the generalisation made above that the male is either quite sterile or is capable of producing the largest litter which can be gestated by the female, and would really come into the category of pre-natal mortality induced by the male.

equally capable of fertilising ova as normal ones). Actually, as shown in the previous section, the size of litter produced during the first four days after irradiation is less than normal—5.54 as compared with the normal of 6.34. If this is explained by the foregoing possibility it would appear that about 12 to 13 per cent. of the spermatozoa were affected. On this hypothesis, however, it is impossible to explain the rise in the male proportion during the first four days, unless it were that the 12 to 13 per cent. of affected spermatozoa were all female-producing,\* and there is no reason why this should be so. It is true that there appears to be a size difference between the X- and Y-spermatozoa, and if this difference extends to the centrosomes the X-spermatozoa would have a greater chance of being disabled. Such an assumption regarding the centrosomes is quite unwarrantable, and, in any case, no such differential vulnerability on the scale demanded by this line of argument can be supposed to exist. In the same way any extension of Crowther's theory seems to lead nowhere when applied to the question of the influence of irradiation on the immature spermatozoa and spermatocytes.

The fact, however, that Crowther's theory does not appear to cover the results under review does not mean that such results offer any contradiction to the theory. Some entirely different factor may be operative.

It should be noted, however, that the histological findings discussed in a previous section does seem to be in contradiction to Crowther's theory. On his view even a sub-sterility dose of X-rays would disable a number of centrosomes, and thus reduce to a certain extent the mitoses in the tubules. In contrast to this expectation the tubules were found to be in a state of great activity, as shown by the enormous number of spermatocytes present

(3) A second possible concept is that some of the spermatozoa present in the epididymis during irradiation receive a lethal dose quite irrespective of the centrosome. It is obvious from both Regaud's and my own observations that all the spermatozoa do not get a lethal dose with the exposures used, but it is not impossible that some may. In this case, since the larger spermatozoa would presumably tend to receive a greater irradiation, the difference in size between the X- and Y-spermatozoa would constitute a possible basis for a differential mortality.

In measuring the head lengths of mouse spermatozoa it was found (Parkes,

\* Given 100 spermatozoa, 50 male producing and 50 female producing, the removal of 12 female producing would leave the ratio 50 : 38, or 56.8 per cent. of males, which, allowing for the slight normal excess of males, brings the percentage to approximately the same figure as that found for births during the first four days after irradiation



14) that the two modes of the frequency polygon were located at respectively 6.2 u. and 6.6 u. a difference of 0.4 u. and a ratio of 0.91 : 1. If this relation is also taken as representative of the diameter of the spermatozoon head the volume relationship will be approximately 18 : 22.\* Let us assume for the moment that the mortality caused by irradiation is differential between Y- and X-spermatozoa in this ratio. Then if half the spermatozoa were affected, the resulting ratio, allowing for the slight normal excess of males, would approximate closely to the ratio observed for the four days after irradiation.† This calculation is, of course, based on a number of assumptions, but it does illustrate a kind of way in which the observed results might be produced.

As regards the number of spermatozoa (if any) eliminated in the epididymis by the irradiation, the following experiments were made with a view to throwing light on this point. Three bucks were irradiated as described in an earlier section for 10 minutes, and 24 hours later the animals were killed by a blow on the head and the epididymus teased out into Ringer-Locke solution at 38° C. The suspensions of spermatozoa produced were examined and compared with that given by a normal male. In no case could appreciable difference in the vitality of active spermatozoa from normal and irradiated bucks be detected. In the same way, the numbers of inactive spermatozoa did not seem to be noticeably different. This experiment was repeated, giving the irradiated bucks double the exposure, an exposure, that is to say, approaching the dosage which leads to sterility. The same results, however, were found on examination 24 hours later. The spermatozoa suspensions from treated and from normal animals presented no detectable differences.

If appreciable mortality had occurred among the spermatozoa at the time of, or just following, irradiation, some trace of this must have been found 24 hours later, both in there being numbers of dead spermatozoa present (they would not be reabsorbed in 24 hours) and in the active ones being relatively fewer than

\* The average diameter of a mouse spermatozoon head is about 2.0 u. and the respective volumes will then be  $6.2 \times \pi \times 0.97^3$  and  $6.6 \times \pi \times 1.03^3$  cubic u.

† Of, say, 200 spermatozoa the normal sex-ratio at birth would be represented by 104 Y-spermatozoa to 96 X-spermatozoa (i.e., approximately, 52 per cent. of male-producing). Now if half these were eliminated in the ratio of 18 Y-spermatozoa to 22 X-spermatozoa, 45 Y-spermatozoa would be eliminated to 55 X-spermatozoa. Of the original 200, therefore, 104 minus 45 Y-spermatozoa to 96 minus 55 X-spermatozoa would remain, i.e., 59 male-producing to 41 female-producing. This would result in 59 per cent. of males being producing, which is very near the ratio found experimentally for the first four days after irradiation (59.4). For this calculation the amount and sex incidence of pre-natal mortality is taken as the same for experimental and normal litters, an assumption which, in view of the slightly smaller litter size of the former, is possibly somewhat erroneous.

normal. Since neither condition was found, it must be supposed that the immediate mortality is very small, and that the disturbance of the normal ratio between X- and Y-spermatozoa is brought about by other means than definite destruction of spermatozoa. The same result could, of course, be produced by affected spermatozoa losing their capacity to fertilise the ovum.

(4) Since the animals are irradiated from below, and the epididymis tends to lie above the testis, it is not impossible that the testis tubules received a greater dosage than the epididymis. Even so, however, it would be expected that the results, while possibly of a different degree, would be of the same order. In any case, since very clear skiagrams could be obtained with the current used, no very great stoppage of rays by the testis could have occurred.

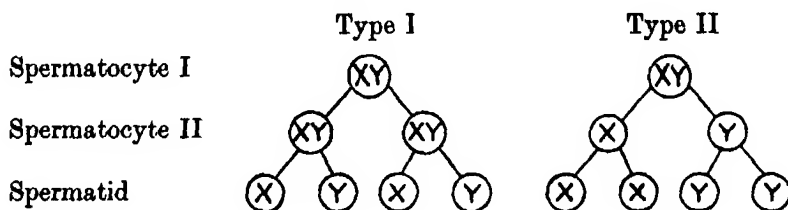
(5) The difference between the periods of male excess and the period of female excess appears, therefore, to be not only an actual empirical result, but also appears to be a true result and not an artefact.

(6) A tempting but improbable possibility is that spermatozoa affected in the epididymis are not killed, but are merely temporarily paralysed, and that more X-spermatozoa are affected than Y-spermatozoa. Subsequent resuscitation of this excess of female-producing spermatozoa would go some way in accounting for the excess of females in the second period. The fallacy here, of course, is that the affected spermatozoa would be ejaculated if coitus took place during the first four days after irradiation and therefore lost, or if coitus did not take place the effect would be neutralised by the other spermatozoa. This leads to another consideration. Since the spermatozoa undoubtedly have the capacity to live for some time in the epididymis, many of the spermatozoa present there during irradiation will, in the absence of coitus during the first period, be available with the spermatozoa produced during the first part of the second period. Now, if the result of irradiation is to accentuate the proportion of male-producing spermatozoa present in the epididymis and to subsequently accentuate the proportion of female-producing spermatozoa, absence of coitus during the first four days would lead to the two effects being mutually compensating, and to the absence of effect during the first part, at any rate, of the second period. Such figures as I have been able to obtain relative to this point do not seem to show that a less excess of females exists during the second period in cases where coitus did not take place during the first period. Either, therefore, spermatozoa present in the epididymis during irradiation do not live long enough, even in the absence of coitus, to produce litters in the second period, or else the neutralising effect is not sufficiently marked to be noticeable.

We come finally to consider the possible origin of the influence apparently

exerted by the X-rays in the spermatozoa produced 5-18 days after irradiation.

(7) Even if irradiation destroys a number of spermatocytes or paralyses their mitotic function, how can such action cause disturbance in the ratio of male- and female-producing spermatozoa which result from their mitotic activity? If the secondary spermatocytes were differential as regards chromatin content some selective basis might be provided, but in mice, apparently, they are not. Taking animals as a whole the reduction divisions may be either of two kinds: the separation of the sex chromosomes may occur either in the divisions of the primary spermatocytes or in the secondary mitoses. The following diagram\* illustrates this:—



In the first type destruction of any number of spermatocytes of either order would result in the elimination of equal numbers of X- and Y-spermatozoa, whereas in the second case destruction of unequal numbers of the two classes of secondary spermatocyte would result in the elimination of unequal numbers of X- and Y-spermatozoa. Mice, however, appear to follow Type I, and thus no basis for selective elimination appears till the spermatids are formed.

It is just as possible to suppose that the spermatids are susceptible to X-rays as it is to suppose that the spermatozoa are—probably they are more so, but one would expect that the differential action would be in the same direction as in the case of the spermatozoa, *i.e.*, the production of a male excess. Actually, the excess is of females. Also, would differential action on spermatids alone lead to the disturbance being prolonged for 18 days after irradiation? In the absence of any accurate knowledge of the time occupied by the life-history of the spermatozoon it is impossible to answer this question definitely, but a negation seems to be indicated. In short, the whole question of how the female excess in the second period originates is most obscure, and were it not for the highly significant nature of the empirical facts the production of the excess might be doubted.

Various hypotheses and considerations relative to the observed results on the

\* Copied from Agar, *Cytology*, London, 1920.





sex-ratio of the exposures used have now been discussed, but it must be admitted that little headway has been made towards a reasonable theory covering the whole of the results.

### VII.—*Summary.*

(1) Various authors have shown that sterility follows prolonged exposure to X-rays in either sex, and several studies have been made on the histology of the irradiated testis.

(2) Experiments described in the present paper deal with the results of sub-sterility irradiations in the male mouse. Such exposures actually appear to stimulate the testis, and peculiarities, notably in the sex-ratio, characterise young from such bucks.

(3) 735 normal mice, bred under conditions similar to those in which the experimental mating were made, gave 379 males and 356 females, a male percentage of  $51.6 \pm 1.24$ .

(4) Young conceived from irradiated bucks within four days of the date of the exposure gave a male percentage of  $59.4 \pm 2.87$ , whereas conceptions during the next fortnight after irradiation gave a male percentage of  $33.6 \pm 2.66$ . After this time the male percentage swings back to  $54.4 \pm 2.27$ , a figure very near the normal.

(5) The litter size was slightly lower than normal during the first four days after irradiation, but afterwards returned to normal.

(6) The origin and significance of these results is discussed.

The mouse colony used for the above experiments was maintained primarily with the aid of a grant from the Government Grants Committee of the Royal Society, to whom my best thanks are due.

### DESCRIPTION OF PLATE 31.

FIG. 1.—Normal mouse testis tubule, showing the 3-5 rows of spermatocytes.

FIG. 2, *a*.—Testis of irradiated animal, showing increase in number of spermatocytes.

FIG. 2, *b*.—Higher magnification of fig. 2, *a*.

FIG. 3.—Similar condition as shown in a different testis.

FIG. 4.—Two tubules showing the beginning of necrotic change.

The Magnification of fig. 2 *a* is 100 times, and of the others 300.

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### *The Effect of Low Temperature on Hens' Eggs.*

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#### INTRODUCTORY.

Colloids when frozen do not as a rule recover their original state on thawing. Stiles\* quotes many such cases, a well-known example being a gel of silicic acid which is separated by freezing and thawing into a mixture of water and solid flakes of acid. Similarly, when a solution of chlorophyll in water is frozen slowly, the chlorophyll on thawing is found to be aggregated into large flocks which slowly settle to the bottom. Recovery of state, however, takes place in certain systems, not necessarily the simplest, if the rate of freezing be high enough. The solution of chlorophyll furnishes an example. If it be frozen in liquid air it completely recovers its original state on thawing.

Obviously the end temperature does not matter because it may be said with

\* "Special Report No. 7 of the Food Investigation Board." Published by H.M. Stationery Office. (1922.)

certainly that the frozen mass could be kept at the temperature of liquid air indefinitely without losing the capacity of reforming a solution on thawing. There must therefore exist a certain limited range of temperature within which alone the process of desolution can occur. Let us call this the critical range. The peculiar feature of any temperature within the critical range  $\theta_1 - \theta_2$  is that, if the system be kept at that temperature for a critical length  $T$  of time, changes take place which are not reversed on thawing.

From what is known of the colloidal state, we may be certain that  $T$  is a function of the form of the cooling curve and

$$T_{\theta} = f\left(\frac{d\theta}{dt}, \frac{d^2\theta}{dt^2}, \dots\right)$$

where the function on the right refers not only to the temperature  $\theta$  but to the whole range  $\theta_1 - \theta_2$ .

The study of egg yolk especially, and to a lesser degree of egg white, is of interest in that it brings out with diagrammatic clearness the fact that the rate of thawing may be as important as the rate of freezing; indeed, the final state depends upon the whole form of the temperature cycle. Moreover, a definite cause, namely, a redistribution and change of state of the chief protein of the yolk, can be assigned to the most marked change which exposure to low temperature brings about. This, and the changes of volume which occur during the temperature cycle, are discussed in the first part of the paper.

The definiteness of the results seemed to warrant an attempt to find a relation between the temperature cycle and the life of the egg. The outcome of the attempt is discussed in Part II.

#### PART I.—PHYSICAL AND CHEMICAL CHANGES.

*The Structure of Egg Yolk.*—Under the microscope, fresh yolk is seen to be composed of rounded masses varying in diameter from  $25\ \mu$  in the outer portion of the yolk to  $150\ \mu$  towards the centre. They are composed of a fine ground substance containing drops of fat which vary in size. The general appearance suggests that these masses make up the whole yolk. In other words, there is no quantity of fluid in which they float. Each mass is semi-fluid, and of a very delicate structure, so that they are not readily detected, owing to the ease with which they break down.

If the yolk of a fresh egg is fixed in Müller's fluid, it after a time crumbles to pieces, and under the microscope reveals a mass of polygons each distinct in itself. These correspond to the yolk masses already described, and they take



on their polygonal shape during fixing. Yolk which has been frozen at any temperature and thawed, behaves quite differently. It becomes horny in the fixing fluid and sections are easily prepared from it. This difference, however, in the behaviour on fixation of a fresh and frozen yolk has nothing to do with the irreversible changes described later. It results simply from the rupture of the yolk masses on freezing. Consequently if fresh unfrozen yolk is well stirred and then fixed, it behaves exactly like frozen yolk and fixes as a continuous horny mass.

*The Freezing Point and Cooling Curves of an Egg.*—Thermo-electric measurements show that the yolk and white of a new-laid egg have different freezing points, that of the former averaging  $-0.65^{\circ}\text{C}$ ., and the latter  $-0.45^{\circ}\text{C}$ . This figure for egg white has also been given by Atkins.\* Moreover, he found that  $\Delta$  for the whole blood of the common fowl lay between the limits 0.60 and 0.66; in other words, it is isotonic with the yolk. Once, however, the yolk leaves the oviduct of the hen, and becomes surrounded by the first layer of white, its freezing point will tend to become equal to that of the white, and eventually equilibrium will be attained. This, however, takes a considerable time and at  $0^{\circ}\text{C}$  is only realized after many months' storage.

Although the freezing temperature of an egg lies between  $-0.45^{\circ}$  and  $-0.65^{\circ}\text{C}$ , whole eggs can readily be cooled below this temperature without any actual freezing of the contents. In the course of this work whole eggs have been stored unfrozen at  $-11^{\circ}\text{C}$ . for periods as long as a week. Such eggs when brought back to normal temperatures show no ill effects and are indistinguishable from fresh.

Fig. 1 shows the cooling curves at the centre of a mass of 30 grams of egg yolk cooled in brine at various temperatures. The supercooling of egg yolk is revealed in curve IV, where separation of ice begins at X. The temperature reached without freezing in this particular experiment was  $-6^{\circ}\text{C}$ ., whilst the yolk of curve V remained unfrozen until the brine temperature was  $-7.4^{\circ}\text{C}$ . The cooling curves for egg white are more or less identical with those for yolk and showed practically the same degree of supercooling.

*The Changes which take place when an Egg is Frozen.*

*The White.*—The changes in the white are not so striking as those to be described later, which take place in the yolk. At all temperatures of freezing there is an increase in the liquid portion with a corresponding decrease in the viscous part. It is probable that there is a separation of water from the latter

\* 'Proc. Roy. Dublin Soc.,' vol. 12, p. 123 (1909).

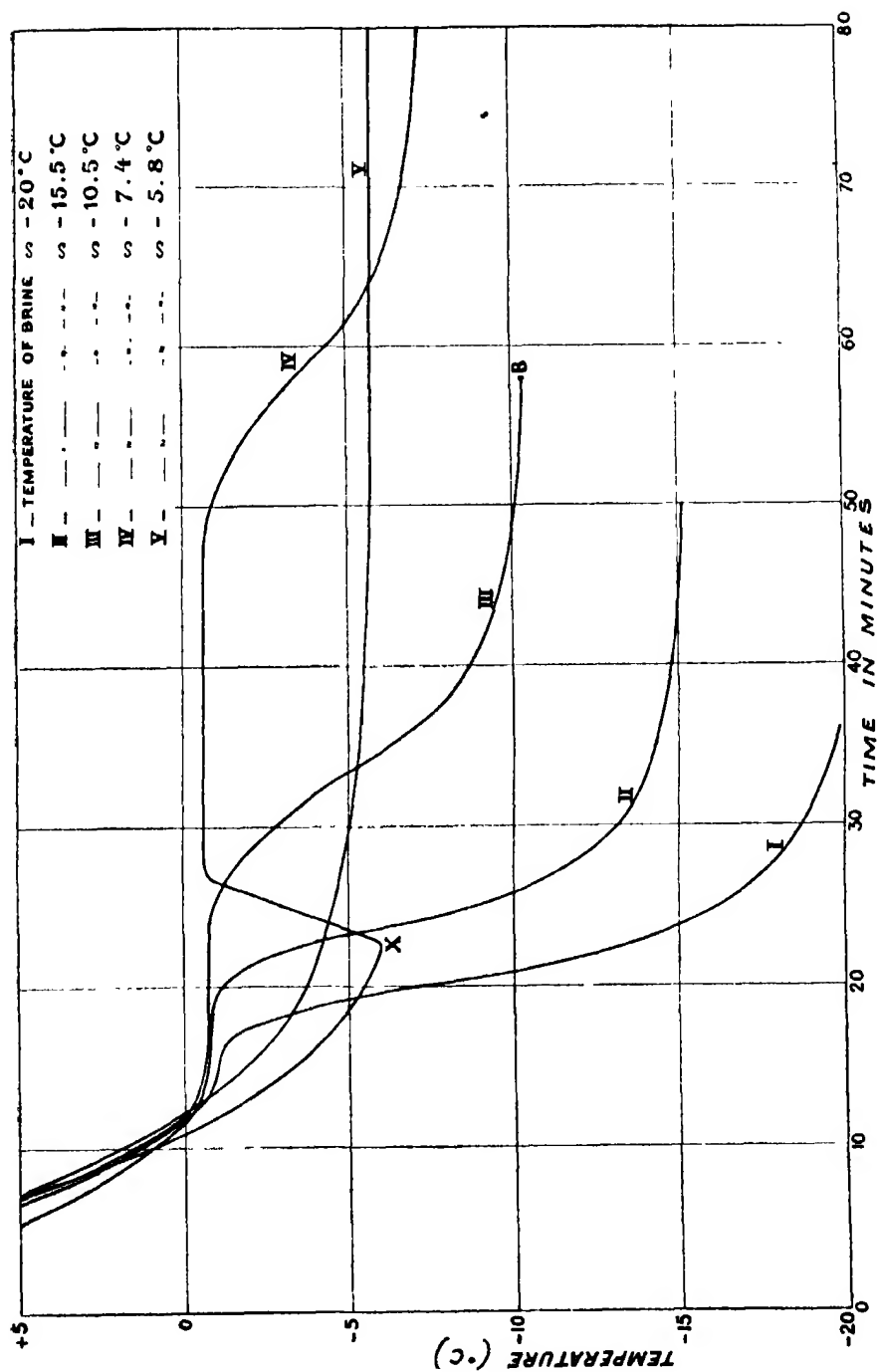


FIG. 1.

on freezing which, not being completely re-absorbed on thawing, goes to increase the net amount of the more fluid portion of the white. This is analogous to the transference of water to the outside of the muscle fibre during freezing, and is a general characteristic of jellies. When, for instance, small masses of gelatin gel are frozen, there is a separation of water, which accumulates on the outside so that it forms a shell of ice. One factor governing the extent of the ice separation with gelatin gel is the temperature reached during the freezing process, and this is also true of white of egg. In the latter case it appears to be the only factor, the freezing and thawing rates playing no perceptible rôle in determining the changes. This is illustrated by the following experiment:—Thirty new-laid eggs from the same source were divided into five lots of six. Of these, (a) six were kept as control and the others frozen (b) in air at  $-3^{\circ}\text{C}$ , (c) in air at  $-16^{\circ}\text{C}$ , (d) in brine at  $-25^{\circ}\text{C}$ ., (e) in air at  $-3^{\circ}\text{C}$ , and then transferred to  $-16^{\circ}\text{C}$ . They were all stored at  $-3^{\circ}\text{C}$ . for 24 hours before thawing, when the relative proportion of the liquid portion of the white was determined by straining through a millimetre mesh. The following results were obtained:—

(a)	Percentage of fluid portion	= 42.
(b)	„ „ „	= 50.
(c)	„ „ „	= 67.
(d)	„ „ „	= 62.
(e)	„ „ „	= 65.

It is clear from these figures that it is the temperature reached, as distinct from the freezing rate, which controls the bulk of the change. Precipitation or coagulation was met with only when the white had been stored in the frozen state for long periods. Thus, after four months' storage at  $-3^{\circ}\text{C}$ . the viscous portion of the white revealed distinct signs of coagulation in the form of white fibres

*The Yolk.*—The changes in the yolk are striking and have been investigated in greater detail. When frozen within the temperature limits  $-0.65^{\circ}\text{C}$ . and  $-6^{\circ}\text{C}$ ., normal fluidity is regained on thawing. If, however, the temperature be carried below  $-6^{\circ}\text{C}$ . and the yolk kept at the lower temperature for a sensible time, it is found on thawing to have changed permanently into a stiff paste-like putty. This change occurs even though the yolk be first of all frozen within the labile limit  $-0.65$  to  $-6^{\circ}\text{C}$ . For example:—Eggs were frozen solid at  $-3^{\circ}\text{C}$ . One moiety was then transferred to a chamber at  $-11^{\circ}\text{C}$ . and left there for seven days. They were then restored to  $-3^{\circ}\text{C}$ .

for seven days, and finally all the eggs were thawed at  $15^{\circ}\text{C}$ . Those eggs which had remained at  $-3^{\circ}\text{C}$ . for 14 days were found to have fluid yolks, whilst those which had been exposed to  $-11^{\circ}\text{C}$ . had pasty yolks. It was proved that the eggs were actually frozen during the first exposure to  $-3^{\circ}\text{C}$ . by taking some at random and sawing them in half. Moreover, as the eggs used were new laid, the shell in all cases had cracked.\*

The critical range for this change in the yolk of hens' eggs begins, therefore, at  $-6^{\circ}\text{C}$ ., i.e.,  $\theta_1 = -6^{\circ}\text{C}$ . This is well below the freezing-point, namely,  $-0.65^{\circ}\text{C}$ . We have therefore the remarkable fact that the change of state can occur when the yolk is frozen so solid as to be capable of being sawn through. Not only is this the case, but freezing—that is, the actual conversion of a large portion of the contained water into ice—is necessary. Eggs are readily supercooled, and have been kept supercooled as low as  $-11^{\circ}\text{C}$ . for seven days without the yolks changing to the pasty state.

For the change of state to occur, it is not sufficient merely to expose to a temperature below  $-6^{\circ}\text{C}$ ., a time factor is involved, as the following experiments show :—

(1) A thermocouple was placed in the centre of an average-sized egg, which was then frozen at  $-5^{\circ}\text{C}$ . The egg was then transferred to a chamber at  $-11^{\circ}\text{C}$ ., and the thermocouple registered that temperature in three and a half hours. In other words, it requires approximately four hours for the whole of an egg to attain a temperature of  $-11^{\circ}\text{C}$ . under these conditions. With this as a guide, a number of eggs were first frozen at  $-5^{\circ}\text{C}$ . and then stored at  $-11^{\circ}\text{C}$ . for various periods of time from two hours to a fortnight. All of them were then brought back to  $-5^{\circ}\text{C}$ . before thawing. It was found that the yolks showed increasing hardness and lack of fluidity in proportion to the time of exposure at the lower temperature. Beyond 24 hours and up to 14 days at  $-11^{\circ}\text{C}$ . the eggs were all alike on thawing. Therefore, in order to complete the irreversible change at  $-11^{\circ}\text{C}$ . in the case of a normal yolk, a period of at least 20 hours is needed. In contradistinction to this, eggs have been stored in the frozen state at temperatures above  $-6^{\circ}\text{C}$ . for several months without showing any signs of change to the pasty state.

(2) A portion of egg yolk was frozen in liquid air. On thawing at room temperature it was found to have passed into the pasty state, but if thawed

\* It may be mentioned in passing that when whole eggs are frozen, the shells are cracked by the expansion of such of the water of the contents as is changed into ice. Cracking may be prevented by partially drying the eggs. If water be lost to the extent of 4 per cent. of the weight of an egg when new laid, it can be safely frozen at any temperature.

rapidly in mercury at 30° C. it completely regained normal fluidity. This proves that the change of state under discussion may occur either during the fall or during the rise of temperature, and takes place in neither if the rate of change of temperature is high enough.

It may be noted, in passing, that pasty yolks which had been frozen in liquid air and thawed at room temperature were finer in texture than yolks which had been frozen slowly.

An indication of the nature of the irreversible change is to be found in the fact that it occurs only when the material is frozen. Egg yolk is a colloidal complex containing in solution proteins, fats, lecithin, and about 1 per cent. of salts. In the frozen state, below — 6° C., there will still be unfrozen a considerable amount of water containing in solution the dissolved salts. As the frozen state is essential to the irreversible change it seems reasonable to suppose that it occurs in the concentrated solution, and the fact that the upper limit of the critical range, namely — 6° C., is well below the freezing point shows that a critical concentration is necessary.

According to Plimmer\* the most abundant protein of egg yolk, vitellin, is present mainly in association with lecithin, and the complex, lecitho-vitellin, is soluble in a 10 per cent. solution of sodium chloride, and is thrown out of solution on the addition of water. The freezing point of 10 per cent sodium chloride is — 6° C. It is probable, therefore, that below this temperature lecitho-vitellin is dissolved by the concentrated salt solution which is formed.

It is well known that once lipins are thrown out of solution from systems such as yolk or blood serum, it is impossible to reform the original state. Blood serum, for example, is totally incapable of re-dissolving the cholesterol esters which it normally contains once they have been precipitated. A sample of lecitho-vitellin was prepared according to the method of Plimmer, and a small portion dissolved in the minimum concentration of sodium chloride possible and frozen in solid carbon dioxide (*i. e.*, below the eutectic temperature of sodium chloride). On thawing there was a white cloud of lecitho-vitellin which never re-dissolved. Another portion of the freshly-prepared complex was placed touching some fresh egg yolk, and observed under the microscope. There was no sign of solution. Lastly, a small quantity of the lecitho-vitellin, well mixed with fresh egg yolk and allowed to stand for a few hours, was brought down again on centrifuging.

The irreversible process in egg yolk may therefore be ascribed to solution of the lecitho-vitellin by the concentrated salt present in the frozen yolk below

\* R. H. A. Plimmer. 'Practical Organic and Biochemistry.'

— 6° C., and its ultimate precipitation on thawing. The time factor apparently required for complete irreversibility at any particular temperature below — 6° C., for example, 20 hours at — 11° C., does not, however, correspond to the time required at that temperature for the complete solution of the lecitho-vitellin, but rather to the time required for the lecitho-vitellin to be thrown out of its normal state in unfrozen yolk. Its solution follows later.

Trial was made of the influence of other substances on the irreversible change. It is well known, for example, that some plants can withstand the effect of frost better than others, and this, as Armstrong\* points out, has been attributed to the presence of a large amount of sugar. Furthermore, plants adjust themselves to the difference in summer and winter conditions by this means, so that whilst a sudden unexpected frost may kill the plant, it can withstand very low temperatures in winter without injury. With this in mind the effect of sucrose in varying amounts on egg yolk was examined, and in all cases it inhibited the irreversible change so that in the presence of 10 per cent. sucrose egg yolk suffers practically no change in fluidity and appearance after being frozen at — 11° C. The exact reason for this is unknown. Shrivastava,† etc., however, explain the protective action of sucrose (and sugars in general) towards colloidal sols on the assumption that a definite percentage of the sugar, depending on its concentration, is adsorbed by the colloid. The theory is supported by measurements of the optical rotation of colloidal solutions of cadmium, antimony and arsenic sulphides containing sugars from which it appears that the adsorbed sugar is optically inactive. The process of adsorption therefore destroys the asymmetry of the sugar molecule.

*Changes in Volume*—The irreversible changes in egg yolk have also been examined by following the volume changes.

The dilatometer used was of the type recommended by Foote and Saxton,‡ and the egg yolk was introduced into the apparatus in the manner they describe. Above the yolk was pure liquid paraffin (sp. gr. = 0.880), the upper level of which was in the capillary stem, the latter being calibrated to 0.1 cm. The choice of a suitable liquid was difficult and liquid paraffin was chosen because it was the only known liquid freezing below — 11° C. and with little or no solvent action on all the constituents of the yolk. In the dilatometer, of course, any solvent action would be limited to the surface of separation which was less than one square centimetre. Moreover, the bulk of the experiment was carried

\* E. F. Armstrong. 'The Simple Carbohydrates and Glucosides.'

† 'J. Phys. Chem.,' vol. 29, p. 166 (1925).

‡ 'J. Amer. Chem. Soc.,' vol. 39, p. 1103 (1917).

out at low temperatures. In a preliminary experiment at normal temperatures 50 gm. of egg yolk and 50 gm. of liquid paraffin were periodically shaken together (thereby greatly increasing the surface area between the two phases) for four days. The paraffin after being decanted gave no test for water with anhydrous copper sulphate, and yielded no residue on ignition. Another portion when ignited with sodium nitrate and sodium carbonate showed only a trace of phosphorus in its reaction with ammonium molybdate. It seems clear therefore that, as far as this particular type of experiment is concerned, the paraffin may be considered to have no sensible action on the yolk.

The expansion and contraction of the liquid paraffin between  $+15^{\circ}$  and  $-11^{\circ}$  C. was perfectly regular, with a constant coefficient over the whole range of  $7.74 \times 10^{-4}$  (relative to glass). Curve P (fig. 2) shows the volumes as given by the capillary height of 36.44 gm. of liquid paraffin at different temperatures in the complete cycle  $15^{\circ}$  C.  $\rightarrow -11^{\circ}$  C.  $\rightarrow 15^{\circ}$  C. (1 cm. of capillary = 0.01774 c.c.). The paraffin was moved slowly through the various temperatures, and was kept at  $-11^{\circ}$  C. for nine days. The cooling and warming curves are seen to be coincident. This curve shows the complete absence of capillary "creep" under the experimental conditions.

Fig. 2 also records the volume changes of 11.73 gm. of yolk and 20.00 gm. of liquid paraffin in a capillary, 1 cm. length of which had a volume of 0.01730 c.c. No attempt has been made to calculate the volume changes of the yolk alone. The variations in volume shown in this curve will be the sum of the changes in volume in (1) the liquid paraffin, (2) the glass container, (3) the air dissolved in the yolk, and (4) the yolk itself. Curve P however shows that the sum of (1) and (2) have a linear relation to temperature and the curve is completely reversible. Their effect therefore can be neglected. It is assumed that the contraction and expansion of dissolved air can be similarly neglected. Any departure from a simple linear relation in these volume temperature curves is therefore due solely to changes taking place in the yolk.

The first important point to be observed is that on cooling the yolk below zero the volume changes cease to be linear, approaching B instead of A'. The mass remained super-cooled until a temperature of  $-7^{\circ}$  C. was reached, at which temperature it froze. Before thawing it was stored at  $-11^{\circ}$  C. for 3 days (F on the curve), to permit of the irreversible changes already noted to take place. On returning to  $-7^{\circ}$  (point G) the volume was found to have increased slightly, but on thawing, the yolk showed a considerable diminution in volume, and the thawing curve between  $0^{\circ}$  and  $8^{\circ}$  C. was not parallel to the cooling curve between the same temperatures. As a result, therefore, of the

irreversible change the egg yolk had (1) altered in appearance, (2) decreased in volume, and (3) possessed a different coefficient of expansion

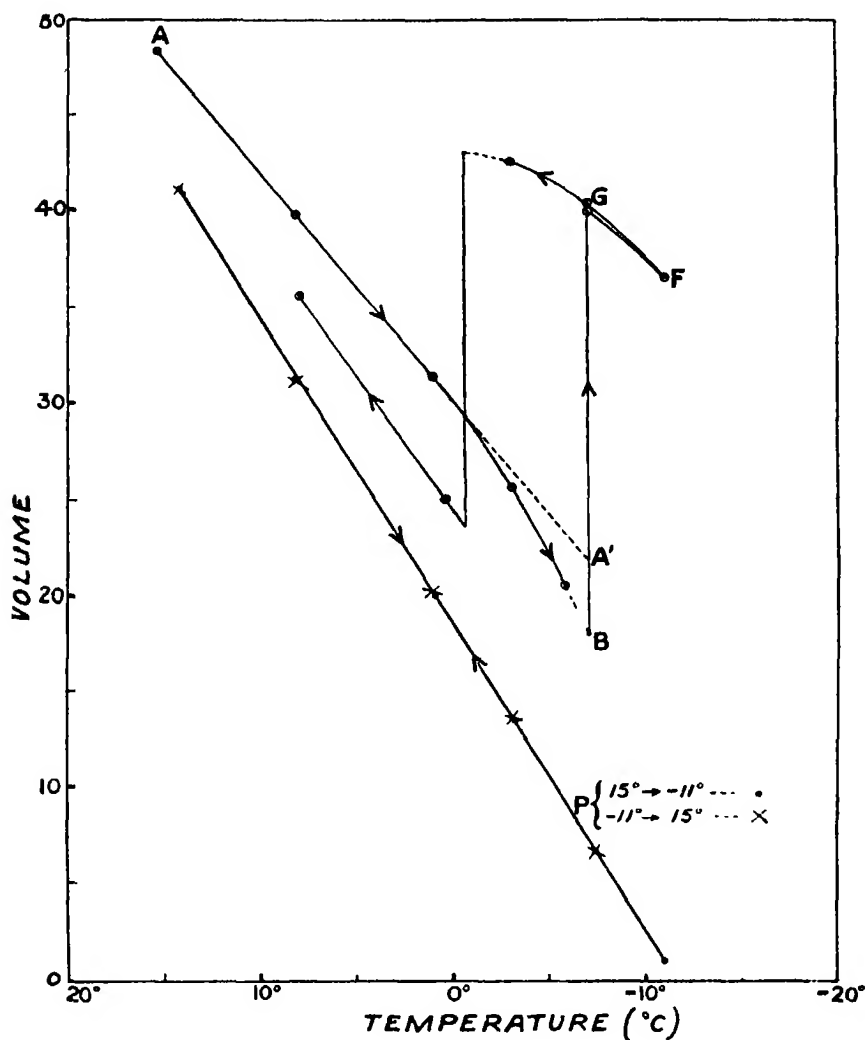


FIG. 2.

The first point to elucidate was whether the volume changes below zero in the unfrozen state were reversible on warming. In this experiment the yolk was cooled down to  $-5^{\circ}\text{C}$ . (B in fig. 3), and then brought back to  $+1^{\circ}\text{C}$ . (C). It will be observed that C does not lie on AB. The change, whatever it may be, is therefore irreversible. The yolk was next frozen and cooled down to  $-11^{\circ}\text{C}$ . and stored at this temperature for 9 days before thawing. A



remarkable feature of the thawing curve FGH is that at  $-7^{\circ}\text{C}$ . the volume is actually less than before storage at  $-11^{\circ}\text{C}$ . With another dilatometer the same phenomenon was encountered. The results are tabulated in the following table.

Table I.

Dilatometer.	Length of storage at $-11^{\circ}\text{C}$ .	Decrease in volume at $-7^{\circ}\text{C}$ . after freezing (i.e., EG) per 1 gm. of yolk.	Total decrease in volume at $0^{\circ}\text{C}$ . after thawing per 1 gm. of yolk.	Percentage of total water freezing out at $-7^{\circ}\text{C}$ .
1 = fig. 2	3 days	+ 0.00015 c.c.	0.0084 c.c.	75.6
2 = fig. 3	9 "	- 0.00082 "	0.0062 "	76.0

### Discussion.

In explaining these results the real difficulty is lack of knowledge of how the various constituents are held in fresh egg yolk. Any explanation can, therefore, be only in the nature of a suggestion.

The important features of the curves are .—

- (1) In the region of supercooling there is a permanent decrease in volume of the yolk ;
- (2) In the frozen state, the outcome of traversing the cycle  $-7^{\circ} \rightarrow -11^{\circ} \rightarrow -7^{\circ}$  quickly is to cause an increase in volume of the yolk, whilst if traversed sufficiently slowly there is a decrease in volume, there would therefore appear to be, at any temperature within this cycle, at first an increase in volume followed by a decrease ;
- (3) There is a further decrease in volume during thawing, so that the final point K of the curve is well below the beginning at A. It will be noticed that the straight line K J, if continued, falls well below the inflection point D.

The curves present many difficulties, especially in the supercooled region, where the risk of secondary decomposition is too great to permit of the prolonged observation necessary to settle whether steady states have been reached. It will be noticed, for example, that the curve AB and the lines KJ and ED, if prolonged, intersect at D. Could this point D' be reached by prolonged supercooling? If so, then the whole contraction in volume produced by the complete freezing and thawing cycle could take place in the overcooled stage, a conclusion of obvious theoretical importance.

Let us consider first the changes which occur on freezing and in the frozen state—that is, the cycle DE, EF, FGH of fig. 3. When traversed quickly,

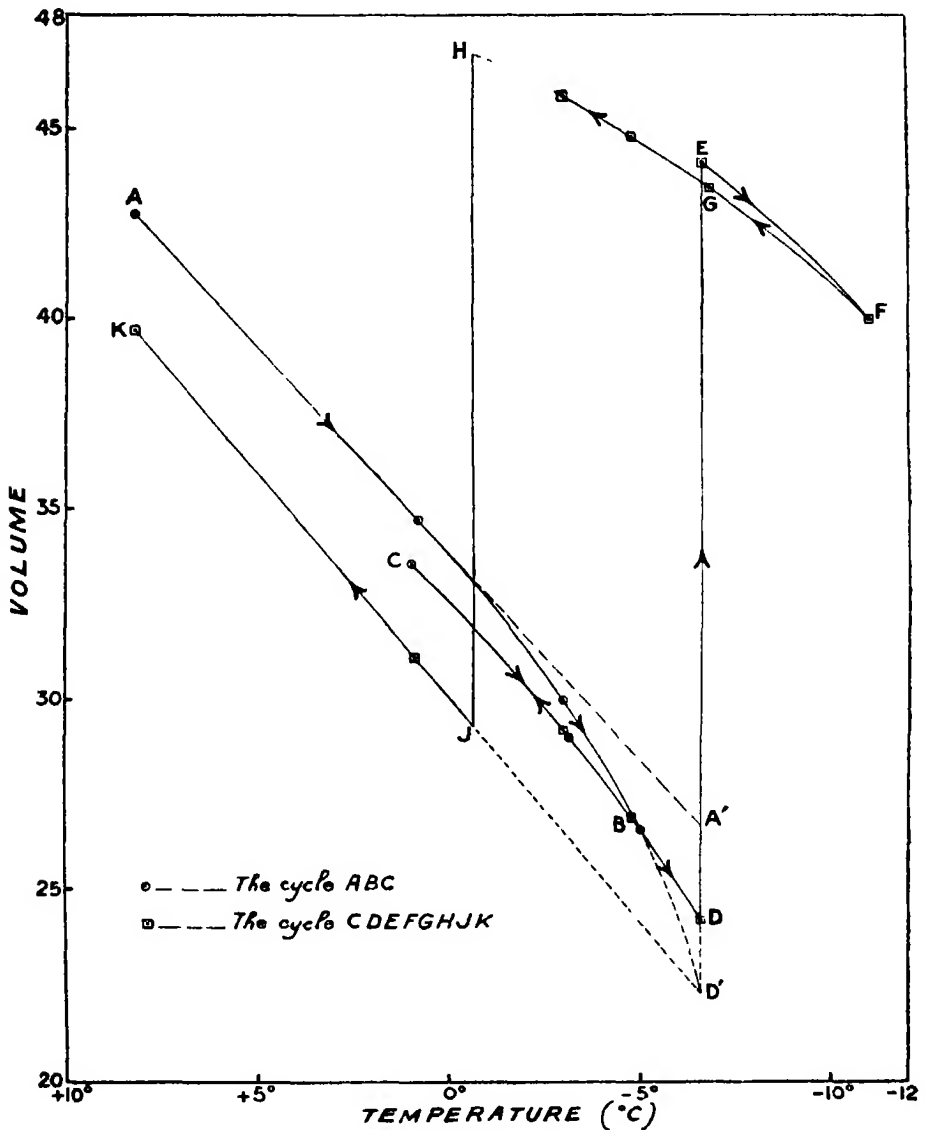


FIG. 3.

there is a slight increase in volume (fig. 2). A permanent increase in volume is the usual result of freezing gels, and is ascribed to the breaking down of capillary structure and the consequent reduction of capillary pressure.

The secondary decrease in volume seen in fig. 3 can, of course, have no such origin. It has, however, been observed that proteins dissolve in water with decrease in volume—for gelatin by Svedberg,\* Taffel† and others, and for caseinogen, egg and serum albumin, and serum globulin by Chick and Martin‡—and the previous discussion in Part I led to the conclusion that the lecithovitellin was withdrawn from its place in the living complex to pass into solution in concentrated salt solution when the frozen yolk fell below  $-6^{\circ}\text{C}$ . It was also pointed out that this process occupied some time. The secondary shrinkage in the cycle under consideration may, therefore, be due to this change.

This hypothesis is unsatisfactory in the appeal it makes to the influences of conjectural capillary spaces. It would perhaps be better simply to class the increase in volume amongst those many effects, such as the over-production of lactic acid in muscle, which have to be referred to a breakdown of an unknown something called "living structure," and leave the decrease in volume, with some degree of confidence, to variations in the state of solution of protein.

The great decrease in specific volume on thawing is shown by the fact that the line KJ, if prolonged, passes well below the point D. Reasons have been given earlier for supposing that the lecithovitellin which is dissolved in concentrated salt solution below  $-6^{\circ}\text{C}$ . is precipitated when the salt solution is diluted by thawing. This should be accompanied by an increase in volume. How then can we account for the observed and great decrease?

The behaviour of dried lecithovitellin in the test tube perhaps furnishes an answer. Dried flakes of the substance suspended in water do not dissolve, but they swell to form a jelly, and it is known that such swelling of protein is accompanied by a shrinkage in the system as a whole.

There remains for consideration the decrease in volume which occurs during simple supercooling. Whatever be the explanation, the discovery of the fact that irreversible changes do take place in supercooling is ample justification for undertaking these troublesome measurements.

The only possible suggestion is that in the supercooled state the quantity of water bound to protein in the living complex is increased.§ If this were all, however, why should such excess not be released when the temperature rose?

\* 'J. Amer. Chem. Soc.,' vol. 46, p. 2673 (1925).

† 'J. Chem. Soc.,' vol. 121, p. 1971 (1922).

‡ 'Biochem. Journ.,' vol. 7, p. 92 (1913).

§ The heat of hydration of all stable hydrates such as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  is exothermic, and therefore the degree of hydration tends to increase as temperature falls.

The change may be irreversible because it involves a breakdown of the living complex with subsequent imbibition of water by the liberated lecitho-vitellin, but this would realise the conditions which are supposed to obtain after actual freezing, when the yolk passes into the pasty state, and simple supercooling has not been found to produce this state. That, however, may be only a question of time for supercooling has never been continued beyond seven days.

#### *Supercooling.*

It has already been said that eggs have been kept as low as  $-11^{\circ}\text{C}$ . without freezing for seven days and without visible change in the yolks, though the dilatometer showed decrease in volume not reversed on warming. Such extreme supercooling is, however, unstable and only rarely encountered. Out of several hundred eggs cooled to  $-11^{\circ}\text{C}$ . at least 99 per cent. were frozen hard in twenty-four hours. It was found, however, that the probability of supercooling was increased by first coating the shell with vaseline. A still better method is to dry the eggs slightly but rapidly to not more than a one per cent. loss in weight, and then slowly cool to  $-11^{\circ}\text{C}$ . Supercooled eggs are easily distinguishable by having the shell uncracked.

#### PART II.—EFFECT OF TEMPERATURE UPON THE LIFE OF THE EGG.

It seems worth while, in view of the positive results discussed in Part I, to investigate the effect of temperature upon the egg as a living organism. For this purpose some particular manifestation of life must be selected, and the capacity for incubation was chosen because this may be said to include all the separate processes such as respiration, heat evolution and growth.

Several workers have investigated the effect of low temperatures on fertile eggs. Colasanti\* found that eggs could be kept at a temperature of  $-7^{\circ}$  to  $-10^{\circ}\text{C}$ . for two hours without losing their fertility, whilst Pictet,† on the other hand, concluded that once eggs are cooled below  $-2^{\circ}$  to  $-3^{\circ}\text{C}$ . the embryo always dies. Later Rabaud‡ claimed to have obtained a number of living embryos after 3 days incubation from eggs stored at  $-18^{\circ}\text{C}$ . for half an hour. He also concluded that the thawing rate does not affect the fertility of the egg, the lowest temperature reached in the cooling process being the significant factor.

Recently Mauro§ has studied the effect of the two temperatures  $+0.5^{\circ}\text{C}$ .

\* Reicherts and Dubois Reymond's 'Archiv für Anatomie' (1875).

† 'Archives des Sciences Physiques et Naturelles' (Sept., 1893).

‡ 'Comptes Rendus,' vol. 128, p. 1183 (1899).

§ 'Atti della Soc. Ital. di Scienze Naturali,' vol. 62, p. 239 (1923).

and  $+14^{\circ}$  C. upon fertile eggs. He found no development on incubating those which had been stored at  $0.5^{\circ}$  C. for 10 days, but "they had started their development, which, however, had stopped." 40 per cent. and 0 per cent. incubations were obtained from eggs stored at  $14^{\circ}$  C. for 20 and 30 days respectively. Mauro also stored eggs at  $0.5^{\circ}$  C. for varying lengths of time up to 10 days, incubating samples of four every day. No incubations were obtained after 72 hours storage at  $0.5^{\circ}$  C. This is surprisingly low, in view of his other experiments, when, with eggs stored for 10 days at  $0.5^{\circ}$  C., some degree of development was found. It is probable that the number of eggs used was insufficient to draw any general conclusions.

In all the work quoted too little attention has been paid to two important points:—(i) The actual temperature reached by the whole egg in any cooling process. Thus as the exact conditions of (say) Colasanti's experiments are unknown it is difficult to decide what temperature the whole egg, particularly the germinal spot, did reach. All we are sure of is that a temperature of  $-18^{\circ}$  C. was certainly not attained in such a short time with the usual freezing means (ii) The number of eggs used.

The conclusions arrived at in this paper are based on experiments with 900 eggs, and the germinal spot certainly did reach the temperature stated.

*Experimental.*—In the first experiment 500 eggs obtained from the one poultry farm (Papworth Colony, Cambridge) were divided into five lots of 100 and placed in five stores, A, B, C, D and E, each of which was at a different temperature (*see* Table II). The eggs, mainly of two types—White Leghorns and White Wyandottes—were packed in cardboard fillers in small whitewood boxes. They were all guaranteed fertile and not more than 48 hours old, and a test on an additional score which had not been stored gave 95 per cent. incubations.

A preliminary experiment had indicated that at low temperatures eggs quickly lose their germinative capacity. Samples were therefore taken out for incubation at intervals of a few hours from rooms A, B and C, whilst from D and E the first sample was taken at the end of 10 days.

The eggs from rooms A, B and C were in all cases placed for two to three hours in room E, where the air was rapidly circulated by a fan. This was done to avoid the rapid warming which would have occurred if the eggs had been transferred from a temperature at or below zero to the incubator. The eggs were incubated in an electrical incubator at a temperature of  $101^{\circ}$ – $102^{\circ}$  F. and were not opened for examination under eight days.

As was expected the eggs gradually lost their fertility at each temperature.

Taking those from room C as an example :—Up to a storage time of 113 hours the percentage incubations was between 100 and 70 per cent. , after 161 hours it had fallen to 30 per cent. and at 237 hours to 16 per cent , beyond which time there was no development in any of the eggs. It was also observed that, in the case of eggs stored at low temperatures and those held for long periods at temperatures above zero, their rate of development was slower than normal, the retardation being greater the longer the storage time. Otherwise the embryos were normal. The limiting time of storage for each temperature at which living embryos were obtained is given in Table II.

Table II.

Storage room	Mean temperature of room.	Limiting time of storage	Actual time whole egg was at temperature of store
A	— 4·6° C	77 hours	47 hours
B	— 2·9	142 „	118 „
C	+ 0·7	237 „	225 „
D	+ 10·4	34 days	34 days
E	+ 16·2	25 „	25 „

It may be suggested that as the eggs were at normal temperatures when received they did not actually reach (say) — 4·6° C. in 77 hours. The approximate time for an average sized egg to reach the temperatures of rooms A, B and C, under the experimental conditions, was determined by making a small hole in the shell of the egg, inserting a thermocouple reaching to its centre, and taking a cooling curve. The egg was placed in the centre of the box and was therefore the last to reach the temperature of the store. It was found that the egg reached — 4·6° C. in 30 hours, — 2·9° C. in 24 hours and + 0·7° in 12 hours approximately. The actual time the egg was at the temperature of each store is given in the final column and is shown in fig. 4 by the dotted line. The number of points on this curve is limited and it is not claimed that the curve is accurate, but rather that it should give a general idea of the results. It was also noted that in general the first eggs to lose their fertility were the White Leghorns. The curve therefore really represents the effect of temperature on White Wyandotte eggs. The curve for White Leghorn eggs would be more or less parallel but slightly lower.

Three important conclusions follow from this experiment.—(i) the life of a fertile egg is considerably reduced by exposure to low temperatures ; (ii) between 0·7° and 16·2° C. there is an optimum temperature for the storage

of eggs so that they maintain their fertility for the maximum period of time ;  
 (iii) this maximum period is determined by the strain of the fowls. Moreover

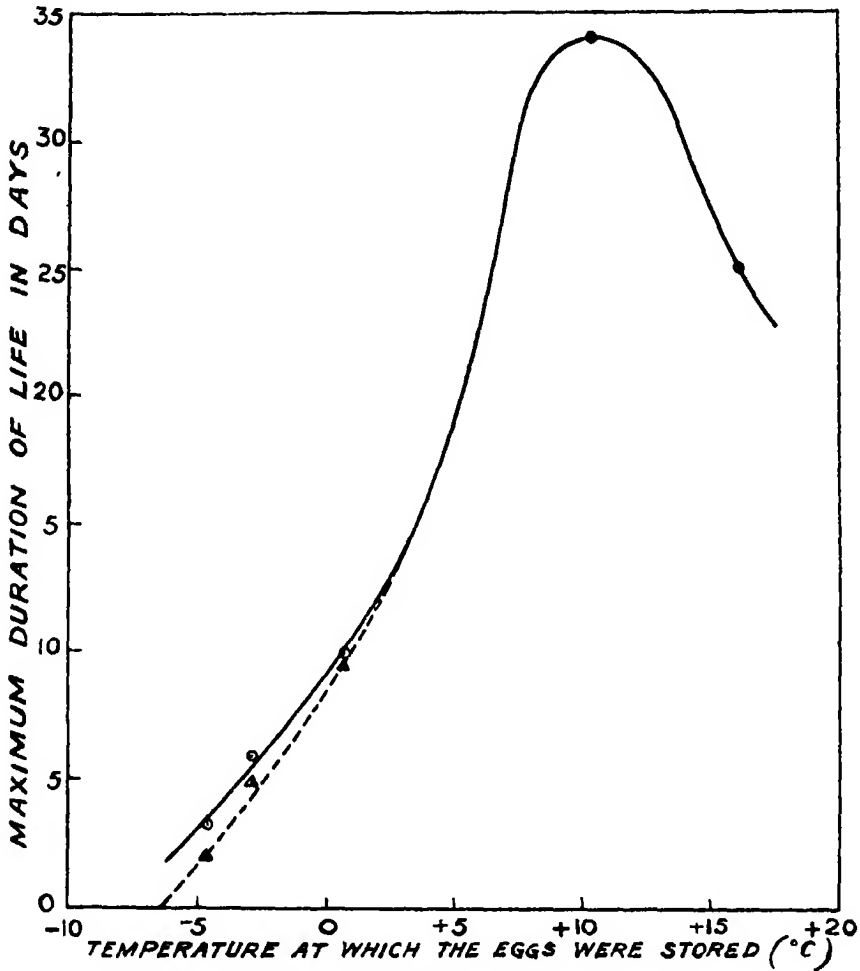


FIG. 4

if one be permitted to extrapolate the dotted portion of the curve it may be concluded that the germinative capacity of a fertile egg is immediately destroyed once it reaches a temperature of  $-6^{\circ}$  to  $-7^{\circ}$  C. The fact that the optimum temperature was so low was quite unexpected. Two months later (the end of July) its existence was again verified in this laboratory by H. P. Hale. This time 200 eggs (100 W.L. and 100 W.D.) were stored at each of the temperatures  $16.2^{\circ}$  C. and  $8.0^{\circ}$  C. (there being no room available at  $10.4^{\circ}$ , the temperature in the earlier experiment). All the eggs were not more than 24 hours

old when received and guaranteed fertile. A sample was taken out for incubation from each room more or less every day, beginning at the 17th day of storage and continued up to 45 days' storage. In this experiment the eggs were incubated for 10 days before examination and only those embryos in which the heart could be seen beating were considered. Partial development is not included. The results fully confirm the earlier conclusions. At  $8.0^{\circ}\text{C}$ . incubations were obtained from eggs up to a limiting storage time of 39 days, whilst at  $16.2^{\circ}\text{C}$ . the maximum time was 29 days. It is quite certain that the optimum temperature does exist somewhere in the region  $8^{\circ}$ – $10^{\circ}\text{C}$ . Fig. 5

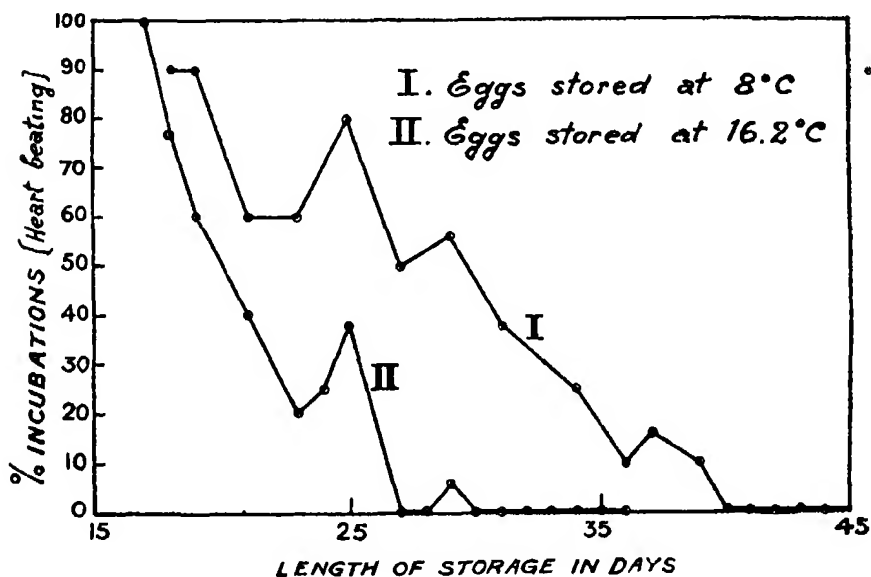


FIG 5

also shows clearly the increased mortality at  $16.2^{\circ}\text{C}$ ., as compared with  $8.0^{\circ}\text{C}$ . throughout the whole experiment, apart altogether from the limiting time of storage.

In this experiment, however, no marked contrast between the behaviour of the two types of eggs was observed, and it could not be said that the Wyandotte eggs were more resistant to cold. On the other hand it was noticed that these eggs had very thin shells as compared with those of the White Leghorns. This, as Riddle\* has shown, tends to weaken the embryo, and it may be that these two opposing factors more or less balanced one another. It will be observed that the limiting time of 29 days at  $16.2^{\circ}\text{C}$ . is greater

\* 'Am. J. of Physiol.', vol. 57, p. 250 (1921).



than the 25 days of the earlier experiment. Enquiries revealed the fact that the feeding of the fowls was different in May and July, in the latter month the quantity of food given to the fowls being greater. This is, therefore, another factor which will have to be considered when discussing absolute values in work of this nature

*Discussion of Results* —To attempt to explain these results is impossible in the light of our present knowledge. The decreasing longevity at temperatures at and below zero is probably a result not altogether unexpected. It has been shown in Part I that egg yolk cooled below zero suffers irreversible changes, which are readily detected by changes in volume. Whether these changes extend to the germinal spot is of course unknown, but it is possible that they are the cause of the decreased duration of life. Bohr and Hasselbalch\* have shown that in the first four days of incubation the changes are endothermic, being accompanied by an absorption of heat. On the basis of the Le Chatelier principle we would therefore expect the eggs to die off more quickly at low temperatures

The explanation of the optimum temperature is, however, quite obscure. Growth in a living organism must involve many physico-chemical reactions, each with its own temperature coefficient, and the existence of at least one optimum temperature favouring longevity might have been expected. That this temperature should be so low is surprising. Knowlton and Starling† have investigated the effect of temperature on the heart beat of the isolated mammalian heart, and find that over a wide range the rate of heart beat varies directly as the temperature. The minimum temperature at which the mammalian heart can beat against an average resistance varies between 23° and 26° C. The maximum temperature lies above 40° and above that temperature irregularities in rhythm begin to appear and after a short time the heart ceases to beat. Pembrey, Gordon and Warren‡ from measurements of the respiratory exchange in eggs during incubation found that during the greater part of the period of incubation (up to the 20th day) the developing chick responds to changes of temperature in a similar manner to that of a cold-blooded animal. Presumably the embryo of the fertile egg prior to incubation is likewise cold-blooded in its behaviour.

Vernon§ has determined the respiratory exchange of the frog at temperature

\* 'Skand. Arch. Physiol.,' vol. 10, p. 149 (1900).

† 'J. of Physiol.,' vol. 44, p. 206 (1912).

‡ *Ibid.*, vol. 17, p. 331 (1894).

§ *Ibid.*, vol. 17, p. 277 (1894).

between  $+2^{\circ}$  and  $30^{\circ}$  C., and found that the relationship between the evolution of carbon dioxide and temperature undergoes a critical change in the region of  $17^{\circ}$  C. Crozier\* has found the same phenomenon with a large number of plants and animals, the temperature, however, being more accurately  $15^{\circ}$  C. No explanation has been put forward to explain this critical temperature, and their experiments therefore afford no obvious help in explaining the results obtained with eggs.

Edwardst† fixes the physiological zero for the hen's egg, *i.e.*, the lowest temperature at which incubation can take place, as  $20^{\circ}$  to  $21^{\circ}$  C. It may be, however, that some degree of incubation proceeds at  $16^{\circ}$  C., giving rise to a system which is not so viable as the embryo of the non-incubated egg. In his paper he quotes the work of Kaestner who found that fresh eggs can be stored at  $21^{\circ}$  C. for three weeks and then incubated. If the eggs have been incubated prior to storage the storage life decreases correspondingly. Thus eggs incubated for one, six and nine days, could only be stored for six, three and two days respectively. In other words one day's normal incubation is sufficient to reduce the storage life of an egg from twenty-one to six days. It is possible that the small degree of development at  $16^{\circ}$  C. is sufficient to reduce its storage life from, say, twenty-nine to twenty-five days, and on the basis of Kaestner's figures, this reduction would be effected by the equivalent of one to two hours normal incubation.

The effect of temperature probably differs from one strain to another, and in this connection it is interesting to note the recent work of Needham‡. He followed the changes in concentration of *D*-inositol during the process of incubation, and found the absolute quantities for Black and White Leghorn eggs markedly different, although the shape of the curves was similar in each case. It is possible that this line of attack will eventually explain the results obtained in the present paper. Having fixed upon a body functioning largely in the metabolism of the developing egg, its behaviour in the egg at various temperatures below normal incubation temperature might profitably be followed.

### *Summary.*

1. After freezing and thawing the white of an egg has become separated into a fluid part and a viscous part, and the increase of the former at the expense of

\* 'J. of Gen. Physiol.,' vol. 7, No. 2, p. 189 (1924).

† 'Am. J. of Physiology,' vol. 6, p. 351 (1902).

‡ 'Biochem. Journ.,' vol. 18, No. 6, p. 1371 (1924).

the latter depends upon the absolute temperature reached and upon the time of exposure to that temperature.

2. Egg yolk after freezing and thawing loses its normal fluidity and passes into a stiff pasty condition. This change occurs only when the yolk is frozen and carried below  $-6^{\circ}\text{C}$ .

3. There is a time factor involved in the change, and the freezing and thawing cycle is also accompanied by permanent changes of volume.

4. Eggs supercooled to temperatures as low as  $-11^{\circ}\text{C}$ . do not suffer any visible change. There is, however, an irreversible change which is manifest by a decrease in volume.

5. The change of state in frozen yolk appears to be due to the precipitation of lecitho-vitellin, so that re-solution does not occur on warming

6. The effect of temperatures between  $-4\cdot6^{\circ}$  and  $16\cdot2^{\circ}\text{C}$  on the fertile egg of the common fowl has been investigated. Below zero the eggs quickly lose their fertility and the present experiments indicate that at approximately  $-6^{\circ}$  to  $-7^{\circ}\text{C}$  the embryo of the egg immediately dies. There is an optimum temperature for fertile eggs in the region  $8^{\circ}$  to  $10^{\circ}\text{C}$ ., at which temperature they maintain their fertility for the longest space of time.

[The above is a Report to the Food Investigation Board of the Department of Scientific and Industrial Research.]

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## *Nuclear Activity in Tissue Cultures.*

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(Communicated by Prof. J. P. Hill, F.R.S.—Received March 12, 1925)

(From the Laboratories of the Imperial Cancer Research Fund, London.)

### *Introduction.*

The extrusion into the cytoplasm of the cell, of a portion of its nuclear content, has been described as occurring during metabolic activity in various types of cells (1, 4). In some cases the material discharged from the nucleus has been regarded as of nucleolar (plasmosomal) origin, while in others it has been said to be derived from the chromatin. As the cells of tissue culture preparations are particularly favourably disposed for cytological examination, it was considered probable that they might show indications of the occurrence of nuclear-cytoplasmic interaction. A study of living and fixed tissue culture preparations was, therefore, undertaken. For this purpose Dr. A. H. Drew kindly placed at my disposal his excellent material, and Dr. A. Begg allowed me to make use of living cultures prepared by him in the course of his research.

### *Previous Work on Tissue Cultures.*

The ideal method for the morphological study of cell activity is, of course, to observe the living cell. This method is, however, beset with considerable difficulties owing to the low refractive index of protoplasm. Both C. C. Macklin and T. S. P. Strangeways have, nevertheless, made observations on living cells in tissue cultures. Macklin (5), working with the cells of the heart of the embryo chick, saw the nucleoli within the nuclei continually undergoing changes in shape, size, and number. He noticed that sometimes the nucleolus came very close to the nuclear membrane to which it appeared to become attached. Amitotic nuclear division, and fusion of the nuclei of binucleate cells, followed by mitosis, were observed by him; also, he studied the pathological process of nuclear fragmentation. T. S. P. Strangeways' observations were made on cultures of the embryonic choroid of the chick (6). He also saw the nucleoli undergoing amoeboid movements and division, and he noted that subsequent fusion of its parts occurred fairly frequently. He followed out the complete mitotic process in his cultures.

W. H. Lewis (2) first described the extrusion of the nucleolus into the cytoplasm in tissue cultures. He studied fixed preparations of endothelium grown *in vitro*. In some of his older cultures, he found that the nucleolus in some way reached the end of the nucleus, and became applied to its membrane. Disintegration took place in this region, the nuclear membrane disappeared, and a vacuole-like area developed in the adjoining cytoplasm, in which the nucleoli came to lie. Lewis, apparently, did not regard this as a normal cytological process, but as being of a somewhat pathological character.

#### *The Cultures Studied, and the Character of their Growth.*

The cells principally studied were fibroblasts of the rat's kidney and skin, in cultures which had been set up in Drew's saline medium, containing embryo extract. Observations were made from both living and fixed cultures, the general characters of which are shown in fig. 4. They were fixed during periods of intensive growth, so as to avoid, as far as possible, degenerative or pathological conditions. Boveri's alcohol-acetic mixture, and Flemming's chrome-osmic-acetic fluid, were the fixatives used, and the iron-alum-hæmatoxylin method was the staining technique employed.

#### *Nucleolar Extrusion in Fibroblasts of the Rat's Kidney.*

What are apparently perfectly healthy cells show evidence of nucleolar extrusion. A cell, the nucleus of which contains a single large nucleolus, closely applied to the nuclear membrane, is shown at *A*, fig. 1. On the opposite side of the nuclear membrane is what appears to be a portion of the nucleolus, which has been discharged into the cytoplasm. It is surrounded by a clear space resembling a vacuole. The three-lobed nucleus of another cell is shown at *B*. Here there are numerous small nucleoli, one of which, it will be noticed, is pear-shaped, and just touches the nuclear membrane. A portion of it has been discharged into the cytoplasm, where it can be seen surrounded by a clear space. To the left of it are two other granules which stain irregularly, and less deeply, and, no doubt, represent nucleolar extrusions which are undergoing disintegration in the cytoplasm. Another type of extrusion of nucleolar material is shown at *C*. This cell originally had two nucleoli; one of these has disintegrated and discharged its contents into the cytoplasm, where they are seen undergoing diffusion.

In each of these cases nucleolar material is apparently discharged into the cytoplasm, where it diffuses.

*Other Nuclear Discharges into the Cytoplasm in Fibroblasts of the Rat's Kidney.*

Besides these cases of nucleolar extrusion, there is to be observed the discharge into the cytoplasm of other constituents of the nucleus, which is possibly

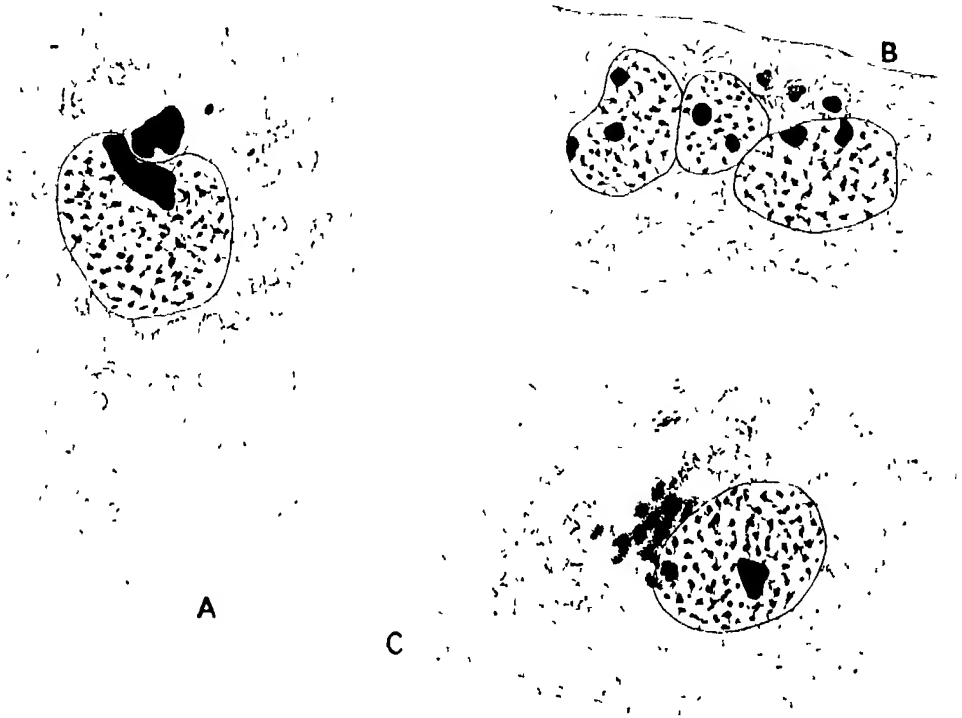


Fig. 1.—Three forms of nucleolar extrusion into the cytoplasm in fibroblasts of the rat's kidney.

an abnormal occurrence. It differs from the so-called nuclear fragmentation described by Macklin (5), in that the nucleus appears to bud off a part of its contents, without undergoing any visible degeneration.

At *A*, fig. 2, is shown what constitutes an intermediate stage between the budding off of a part of the nucleus, and a nucleolar extrusion. It will be noticed that the nucleolus has seemingly given off a particle which is passing out into the cytoplasm, carrying with it other nuclear constituents. A similar, but perhaps little later stage, of this process is shown at *B*. Occasionally there are seen cases in which the nucleus appears to be pouring out a considerable part of its contents into the cytoplasm. At *C* and *D* are shown the nuclei of two such cells. The nucleolus of the former is divided up into several parts, and the lower part of the nucleus, containing some of these, appears to be

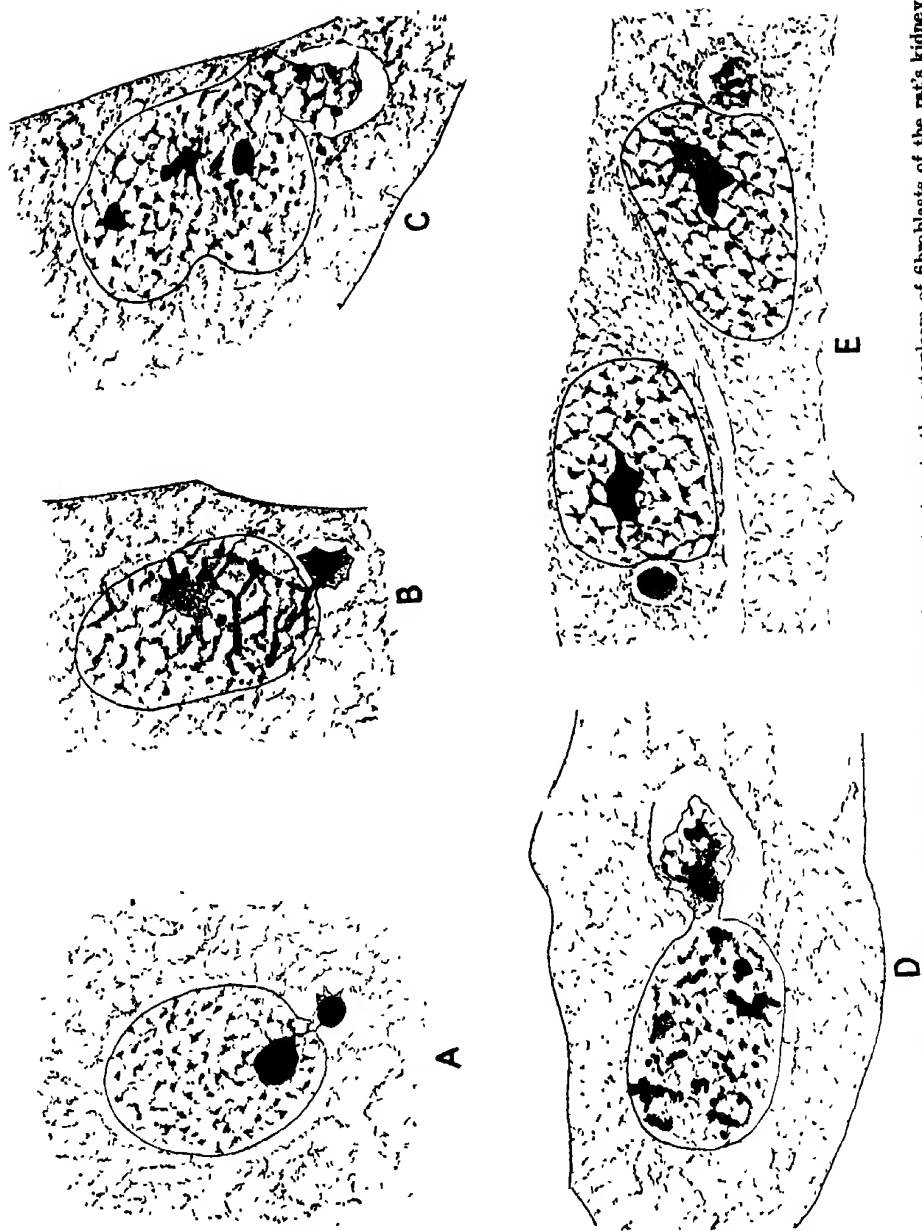


Fig. 2.—Successive stages in the extrusion of a portion of the nuclear contents into the cytoplasm of fibroblasts of the rat's kidney.



FIG 3.—Discharge of a portion of the nucleus into the cytoplasm in amoeboid migratory cells, probably of endothelial origin. The implant of rat's kidney from which these cells had been derived was below these and slightly to the right. A shows a form of nucleolar extrusion; B the budding off of a portion of the nucleus



discharging into the cytoplasm. At *D* the nucleolar content of the nucleus is even more scattered, and there is similar indication of an outward discharge from the nucleus. That part of the nucleus thrown off into the cytoplasm seems to persist for a time, as a kind of secondary nucleus, and then diffuses into the cytoplasm, as is seen taking place at *E*.

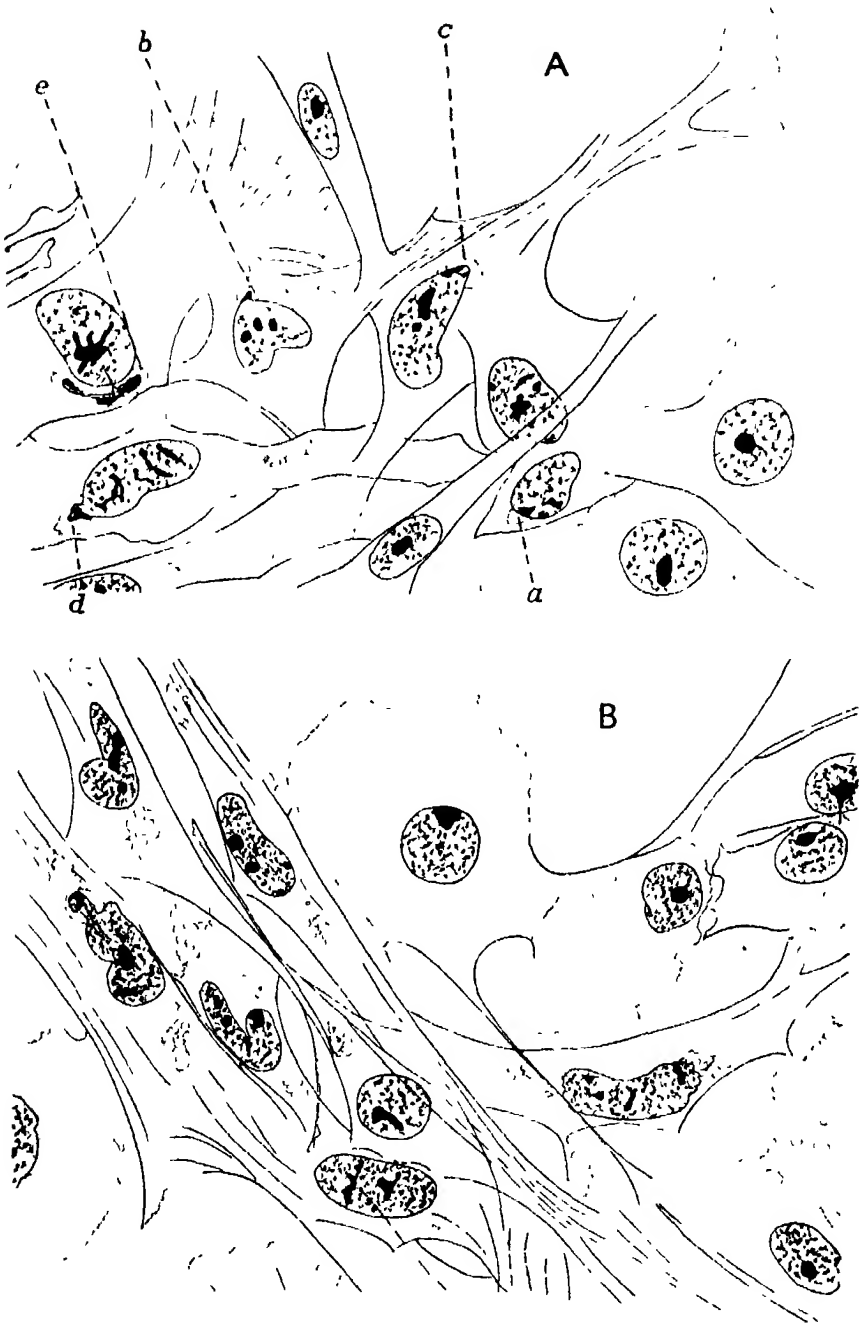
The general appearance of some of the nuclei figured suggests that their peculiar character may be due to shrinkage of the nuclear membrane, brought about by the action of the fixative. The occurrence, however, of small nuclear-like fragments in the cytoplasm is suggestive of their having originated in the manner described.

Certain cells of an amoeboid form, and probably of an endothelial nature, which have migrated outwards from the implant into the medium, also show the same peculiarity. At *A* and *B*, fig. 3, are shown two such cells. In the preparation from which these were drawn the "implant" lay below them and slightly to the right.

It will be noticed in the figures that the nucleus appears to discharge into a vacuole in the cytoplasm. This vacuole may possibly arise before the discharge occurs. What are regarded as the successive stages in the process are shown at *A*, fig. 4, *a* to *e*. At *a* a small vacuole is seen at the side of the nucleus, at *b* a nucleolar particle is bulging into the vacuole, at *c* the bulge is bigger, at *d* the nuclear protrusion is rounding off, while finally its complete separation from the nucleus is seen at *e*, where it is entirely surrounded by the vacuole. Indications of the same process are shown at *B*, of the same figure, where there is also seen a vacuolation of the cytoplasm in the neighbourhood of the nucleus, which is evidently an indication of interaction between the nucleus and cytoplasm.

#### *Observations on Living Cells.*

With the view to studying in the living cell the cytological processes already described, cultures were kept under observation by means of a microscope. The microscope used was enclosed in a warm box devised for this purpose by Dr. J. A. Murray, the Director of the Laboratories, who also assisted me in the carrying out of extended observations. The stage of the microscope upon which the cultures rested was kept constant at 38° C., the normal body temperature of the rat. Fibroblasts were the cells principally studied. They were observed both by transmitted light, and by dark ground illumination, by means of a 2 mm. oil-immersion lens. When transmitted light was used, the cultures were taken from the incubator, and observed directly in the hanging drop



**FIG. 4.**—General character of the growths of fibroblasts of the rat's kidney. *A* shows what are regarded as the successive stages in the extrusion of a part of the nuclear contents into the cytoplasm (*a* to *e*); *B* shows similar activity, and also the vacuolation of the cytoplasm, frequently in the immediate neighbourhood of the nucleus.

cultures ; but with dark ground illumination, the cover-slip with the culture had to be arranged face downwards on another glass slide, on which a drop of the culture medium had previously been placed.

When cells are examined by either of these methods, it is a matter of some considerable difficulty to follow with certainty the nature of the cytological activities in progress. Macklin (5) has discussed this method of study and pointed out its difficulties. He tried various vital staining methods, none of which could be regarded as satisfactory for the purpose. Certain structure in the unstained cell are more or less conspicuous by transmitted light, and Lewis (3) has described the examination of cells by dark-ground illumination, while Strangeways has demonstrated the movement of mitochondria by the same method : yet for critical study the living cell is far from being an easy subject.

In the rat fibroblasts, the nucleolus was at first easily distinguishable as a highly refractile body within the nucleus. It could be seen making slow movements within the nucleus, as has already been described by other observers in cultures of tissues of the chick embryo. Sometimes the nucleolus could be seen approaching the nuclear membrane, and also moving away from it towards the centre of the cell. This later process is shown in fig. 5, which

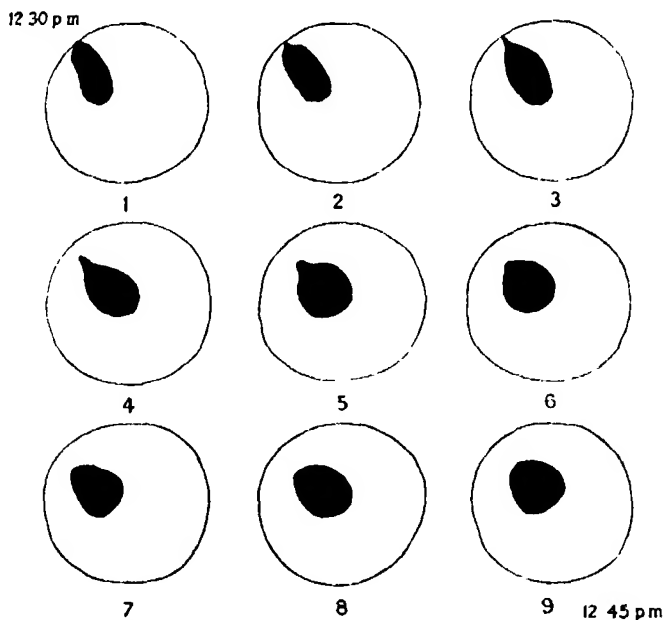


FIG. 5.—Somewhat diagrammatic representation of the movement of the nucleolus, away from the nuclear membrane, towards the centre of the nucleus, drawn from a living fibroblast in a culture of rat's kidney, at approximately equal intervals, over a period of 15 mins.

represents the nucleolus slowly moving away from the nuclear membrane and assuming, at first, a somewhat pear-shaped form, then becoming ultimately rounded off on reaching a more central position within the nucleus. The process figured lasted 15 minutes. Its initial stages were slightly more rapid, and on attaining the final position, stage 9, little movement was seen, although the cell was kept under observation for some time afterwards.

Keeping the cultures on the warm stage caused a remarkable change in the appearance of the nucleolus after a short time. The nucleolus seemed to become gradually more and more indistinct, as did also the nuclear membrane. If in this state the culture was removed, and examined at room temperature, these cell structures became more easily distinguishable. The meaning of these changes is not known, but they introduced a considerable difficulty to the making of observations.

The extrusion of nuclear material into the cytoplasm could not, therefore, be followed with precision, but in fig. 6 is shown what is probably to be regarded

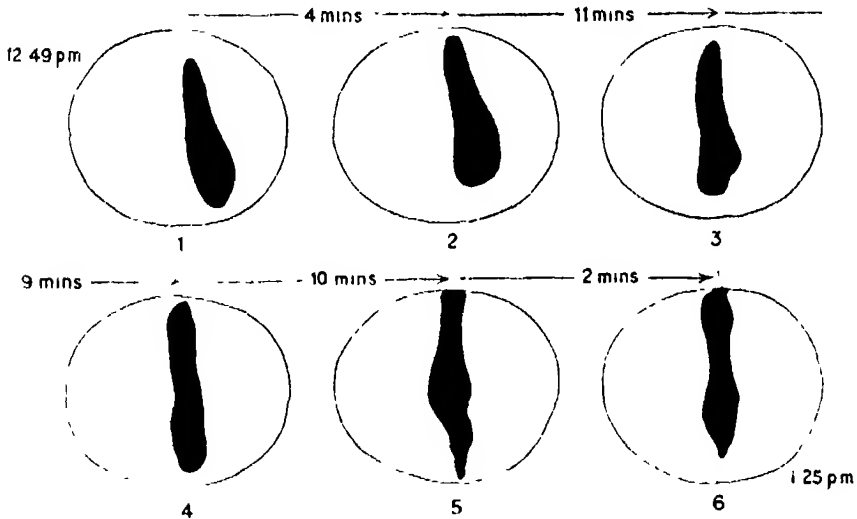


FIG. 6 —Diagram illustrating the movement of the nucleolus within the nucleus, and its application to the nuclear membrane. At "6" is indicated a vacuole in the cytoplasm directly opposite the nucleolus, from which it has apparently originated. The intervals of time elapsing between the stages shown are inserted above the figures.

as a discharge of a part of the nucleolus from the nucleus. In the first place, the nucleolus is an elongated body extending nearly right across the nucleus. At first it showed a slight movement upwards, "2," then a downward movement, "3," followed by a gradual move towards the nuclear membrane in the initial direction, "4" and "5." Finally, the nucleolus became applied to the

nuclear membrane. opposite which a relatively large vacuole made its appearance in the cytoplasm, "6." This vacuole evidently represented extruded nucleolar material. It was impossible to be quite certain whether this vacuole was continuous with the nucleolus through the nuclear membrane, or to determine its relation to the other vacuoles within the cytoplasm, but from a comparison with the fixed and stained preparations the appearance observed is very suggestive of nucleolar extrusion.

#### *General Conclusion*

From a study of fixed and stained preparations, supplemented by the observation of living cells, it is seen that the nucleoli of the fibroblasts of the rat's kidney perform during life slow amœboid movements. Occasionally a nucleolus approaches the nuclear membrane when part of it is discharged into the cytoplasm where it disintegrates. Also, a portion of the nucleus itself may be budded off, persist for a time as a kind of secondary nucleus, and then diffuse into the ground cytoplasm; however, as has been pointed out, this process is possibly of an abnormal character. The extrusion of a portion of the nuclear contents may be preceded by the formation of a vacuole into which the nuclear substance is discharged; but nucleolar extrusions are possibly surrounded later by fluid droplets. It is doubtful, though, to what extent such vacuole-like appearances are due to shrinkage consequent upon fixation.

The frequent occurrence of vacuoles in the cytoplasm, surrounding the nuclear membrane, is also suggestive of interaction between the nucleus and cytoplasm.

#### *Summary.*

1. Fixed and stained cultures of fibroblasts of the rat's kidney were studied for indications of nuclear-cytoplasmic interaction.

2. Extrusion from the nucleus into the cytoplasm of a part of the nucleolus was found to occur. The discharged nucleolar material was surrounded by a vacuole. It ultimately disintegrated and diffused into the cytoplasm (fig. 1, *A*, *B* and *C*).

3. Occasionally the nucleus appears to bud off a portion of its contents, which may persist for a time as a kind of secondary nucleus and then break down. It has been pointed out that this process is possibly an abnormality (fig. 2, *A* to *E*). What probably indicates the successive stages in this process are shown in fig. 4.

4. A similar discharge of nuclear material was observed in cells which had migrated by amœboid movements, far out from the implant, into the culture medium. Such cells are apparently of endothelial origin (fig. 3).

5. Living cultures of rat kidney were kept under observation at the body temperature of the rat, for indications of the occurrence of the processes seen in the fixed preparations. Slow amœboid movements of the nucleolus were seen, and also the movement of the nucleolus towards and away from the nuclear membrane (fig. 5).

6. Since a vacuole has been seen to form in the cytoplasm opposite the nucleolus when it has become applied to the nuclear membrane, it is believed that this is the actual discharge of nucleolar material, or at least its initial stage, corresponding to that figured from the fixed preparations in fig. 1.

7. After cultures had been kept under observation at 38° C. for a time, the nucleolus and nuclear membrane became more difficult to distinguish, but on transferring the culture to a microscope stage at room temperature, these structures were rendered more conspicuous. Such changes in the visibility of the parts of the cell, together with the normal transparency of protoplasm, render the study of the living cell extremely difficult and prevent precise observations being made.

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*Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen.—Part XI. Pulse Rate and Oxygen Intake during the Early Stages of Recovery from Severe Exercise.*

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(Communicated by Prof. A. V. Hill, F.R.S.—Received June 6, 1925.)

(From the Department of Physiology, University College, London.)

At the commencement of muscular exercise the oxygen intake and the pulse rate increase rapidly, but soon attain a steady value depending on the severity of the exertion. In strenuous exercise the intake of oxygen, although large, may not be sufficient to effect the oxidative removal of all the lactic acid formed, and the body goes into "oxygen debt." During the recovery, therefore, from severe exercise, as opposed to that from mild exercise, the rate of fall of the oxygen intake is less rapid than was its rate of increase during the first minute or so of exercise. We find a condition in which the oxygen intake is determined, not by the contemporary requirement of the body in respect of the exercise which it is taking at the moment, but by a "debt" which was incurred during a previous period. At the end of several minutes of violent effort there is a considerable need of oxygen, which is not satisfied completely for a comparatively long time. Thirty seconds after the end of such exercise the need for oxygen is presumably nearly as great as it was during the exertion itself.

The fall in the oxygen intake, therefore, which occurs immediately at the end of exercise is determined, not by any appreciable change in the requirement of the body for oxygen, but rather by an alteration in the mechanism by which it can be supplied. The immediate fall, in fact, of the oxygen intake must be credited largely to a change in the circulation rate of the blood, determined may be by the abrupt cessation of bodily movement, and not to any sudden alteration in the need for oxygen. During the first few moments, therefore, after exercise ends, when the oxygen requirement is still high, we may regard the oxygen intake as some measure of the circulation rate of the blood, and it is natural to compare it with the only other factor which can be continuously recorded for the circulation, namely, with the pulse rate. The simultaneous determination of the pulse rate and of the oxygen intake, during

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the early stages of recovery from severe exercise, has been made in the experiments to be described.

During rest the oxygen consumption is the same whether the subject breathes room air or gas mixtures rich in oxygen [Durig (9), Benedict and Higgins (10)]. This fact has been confirmed by one of us in the subsequent paper. Hill, Long and Lupton (1) have shown that the later stages of the recovery process are not shortened by breathing rich oxygen mixtures. At rest and in the later stages of recovery the oxygen intake is sufficient to meet the demands of the body at the moment. During severe exercise, however, Hill, Long and Lupton (2) have shown, and Furusawa (3) has confirmed, that the oxygen intake is greater when the subject breathes rich oxygen mixtures. the increase may amount to 50 per cent. Part of the increase is due presumably to a better saturation of the blood in its passage through the lungs. Mainly, however, the increase must be due to an increased output of the heart per beat, owing to the better saturation of its coronary blood. It is not caused by an increase in the pulse rate. If a rich oxygen mixture largely increases the oxygen intake during the exercise, it must increase it also during the first few moments of recovery, and in the first minute at any rate, after severe exertion, one may well expect the oxygen requirement to be considerably in excess of the oxygen supply.

The same conditions as obtain during exercise, namely, those of oxygen want, exist also during the first minute or two of recovery. If we may allow the oxygen intake during exercise to be determined by the circulation rate of the blood, we may justly assume the same factor to be the determining agent in the oxygen intake during the initial phases of the recovery process. Later on, when the oxygen need is not so urgent, the coefficient of utilisation of oxygen in the blood may fall, and the oxygen intake be no longer a measure of the circulation rate, as it probably is for the first few moments, and for an unknown period thereafter. A simultaneous determination of the pulse rate gives us a means of approaching the important problem, of how far the output of the heart per beat is altered by the sudden stoppage of the mechanical movements of the body, and by the consequent greater difficulty in the venous return of blood to the heart. Although from the nature of the observations possible under such conditions, it is not possible to calculate exact quantitative results, a study of the data to be presented has brought a conviction that, since the oxygen intake falls off much more rapidly towards its resting value than does the pulse rate, even at a time when there is considerable oxygen need, the output of the heart per beat must be appreciably diminished by the stoppage of the bodily movements which normally aid in the venous return.



It has been shown by Buchanan (4) and others that the heart rate is increased directly exercise commences, the first beat after making the effort being shorter than the one previous. This is not due to an increased supply of venous blood to the heart, since it occurs equally when the limb is ligated. The rapidity of the response suggests the removal of vagal tone rather than an increased sympathetic action [see Bainbridge (5)]. At the commencement of exercise the rise of pulse rate must be a large factor in the rapid increase in oxygen intake, accompanying an increased venous return of blood to the heart. The gradual rise, however, of the oxygen intake to its maximum value is not to be credited entirely to circulatory factors; it must depend largely upon the gradual rise of the speed of the recovery process itself, as the concentration of lactic acid in the active tissues gradually approaches its maximum or its full steady value characteristic of the intensity of the exercise.

*Method.*—For the collection of a rapid succession of samples of expired air we used the Douglas bag technique in the manner described by Hill, Long and Lupton ((1) p. 87, fig 2). The exercise was “standing running” throughout the experiments, the subject moving in time with a metronome. The preliminary exercise before the observation began was sufficiently severe to produce a large oxygen debt. The electrodes for recording the heart rate (see (1), p. 88), and also the mouthpiece were fixed to the subject, after which he exercised until he showed signs of distress. The tap of the first bag was then opened and a sample collected. At the end of 30 seconds exercise was stopped sharply, and at the same moment the tap of the first bag was closed and that of the second was opened. Seven collections were made successively over a period lasting from a moment 30 seconds before till another three minutes after the end of exercise.

The pulse rate was recorded by a string galvanometer from which the magnifying eye-piece was removed. A paper camera was employed and the speed of the paper adjusted to about 1 cm. per second. A narrow slit about 0.75 mm. wide admitted the light into the lens of the camera. Time intervals of  $\frac{1}{2}$  sec. were recorded by a rotating time-marker placed close to the slit. A Jaquet clock marked seconds also to facilitate counting. The electrodes were made of thin lead sheet about  $7\frac{1}{2}$  cm. square; they were covered with cotton wool soaked in 10 per cent. saline and placed one in contact with the back of the right shoulder and the other in relation to the heart's apex. They were held in place by a wide elastic band. The skin was vigorously cleaned with methylated spirit so as to remove grease and cause a hyperæmia. The current was led off through the fibre of the galvanometer in series with a con-

denser of about 50 microfarads capacity. By using this arrangement it is not necessary to compensate for the "skin currents" of the subject, and also, since the rate of change of potential of these currents during exercise is slow, they produce little or no deflection of the fibre of the galvanometer. The electro-cardiogram was taken for the last 15 seconds of exercise, and the first two minutes of recovery. A further 15-second record was taken 3 minutes after the end of exercise. The records were measured by the Lucas Comparator. Measurement was carried out for every 10 beats during the first half-minute and after that for every 20 beats. The results are expressed in beats per minute. The records, although very small, are quite clear, and the error of measurement does not amount to more than one beat per minute.

Four subjects were employed and ten experiments were performed.

Subject K.F., weight 50 kilos., normal oxygen intake (lying), 250 c.c./min.  
average normal pulse-rate 70 beats per min.

„ R.M.S., weight 63 kilos., normal oxygen intake (lying) 200 c.c./min.,  
average normal pulse-rate 85 beats per min.

„ R.J.L., weight 70 kilos., normal oxygen intake (lying) 250 c.c./min.,  
average normal pulse-rate 70 beats per min.

„ J.R.P., weight 59 kilos., normal oxygen intake (lying) 250 c.c./min.,  
average normal pulse-rate 84 beats per min.

*Results.*—In Table I the details of the metabolism determinations for each experiment are shown.

Table I—Respiratory Data

Subject	Nature of exercise	Time of collection of sample	Ventilation L p m	O <sub>2</sub> intake c c /min	CO <sub>2</sub> expired c c /min	Respiratory quotient	Increase of O <sub>2</sub> consumption above resting c c /min
R.M.S. (K) 17.3.25	240 steps per min.  Fore period. 2 m. 5 s	30 sec. during expt	96.4	3,917	3,750	1.2	2,817
		20 „ „ recovery	65.0	2,333	3,636	1.1	2,133
		20 „ „ „	60.2	1,818	2,244	1.2	1,618
		20 „ „ „	46.1	1,232	1,690	1.4	1,032
		30 „ „ „	52.7	1,154	1,702	1.4	954
		30 „ „ „	50.0	960	1,335	1.5	760
		60 „ „ „	43.9	800	1,250	1.4	660
R.M.S. (H) 13.3.25	240 steps per min.  Fore period. 2 m. 5 s	30 sec. during expt.	80.1	2,819	3,270	1.1	2,619
		20 „ „ „ recovery	61.0	2,263	2,502	1.1	2,063
		20 „ „ „	57.8	1,780	2,330	1.3	1,580
		20 „ „ „	46.8	1,320	1,810	1.4	1,129
		30 „ „ „	41.8	1,045	1,540	1.4	845
		30 „ „ „	36.3	932	1,222	1.3	732
		60 „ „ „	29.7	772	965	1.2	572

Table I.—*continued.*

Subject.	Nature of exercise.	Time of collection of sample.	Ventilation L p m.	O <sub>2</sub> intake c.c./min.	CO <sub>2</sub> expired c.c./min.	Respiratory quotient.	Increase of O <sub>2</sub> consumption above resting c.c./min.
R.M.S. (G) 9.3.25	190 steps per min.  Fore period : 2 m. 30 s.	30 secs. during expt.	95.6	3,604	4,190	1.1	3,404
		20 " " recovery	43.2	1,468	1,690	1.1	1,268
		20 " " "	39.5	904	1,375	1.5	704
		20 " " "	41.2	692	1,271	1.8	492
		30 " " "	40.2	603	1,122	1.8	403
		30 " " "	—	—	—	—	—
		60 " " "	39.3	235	632	2.6	35
R.J.L. (I)	240 steps per min.  Fore period : 2 mins.	30 secs. during expt.	47.3	2,767	2,534	0.91	2,217
		20 " " recovery	62.6	2,416	2,605	1.0	2,166
		20 " " "	65.7	1,892	2,480	1.3	1,642
		20 " " "	53.3	1,263	1,722	1.3	1,013
		30 " " "	54.4	1,022	1,485	1.4	772
		30 " " "	27.8	806	990	1.2	556
		60 " " "	19.8	592	544	0.91	342
R.J.L. (D) 12.2.25	200 steps per min.  Fore period : 60 secs.	30 secs. during expt.	39.2	2,575	2,050	0.79	2,325
		20 " " recovery	39.6	2,229	1,960	0.87	1,979
		20 " " "	31.7	1,632	1,570	0.96	1,382
		20 " " "	26.0	1,185	1,110	0.93	935
		30 " " "	23.5	836	917	1.0	586
		30 " " "	19.4	508	647	1.2	258
		60 " " "	14.5	450	496	1.0	200
K.F. (F) 3.2.25	280 steps per min.  Fore period : 2 m. 15 s.	30 secs. during expt.	79.5	2,487	2,790	1.1	2,237
		20 " " recovery	57.4	2,188	2,210	1.0	1,938
		20 " " "	52.9	1,707	1,935	1.1	1,457
		20 " " "	43.7	1,339	1,585	1.1	1,089
		30 " " "	37.7	803	1,330	1.5	553
		30 " " "	28.5	672	947	1.4	422
		60 " " "	19.2	523	662	1.2	273
K.F. (J) 16.3.25	240 steps per min.  Fore period : 3 mins.	30 secs. during expt.	63.1	2,315	2,278	0.98	2,065
		20 " " recovery	47.9	1,973	1,882	0.95	1,723
		20 " " "	38.4	1,416	1,450	1.0	1,166
		20 " " "	29.9	876	1,100	1.2	626
		30 " " "	25.8	694	890	1.2	444
		60 " " "	17.5	521	587	1.1	271
J.R.P. (B) 9.2.25.	240 steps per min.  Fore period : 60 secs.	30 secs. during expt.	—	1,816	2,117	1.1	1,566
		20 " " recovery	—	1,525	1,830	1.2	1,275
		20 " " "	—	1,265	1,809	1.1	1,015
		20 " " "	—	978	1,635	1.8	758
		30 " " "	—	—	—	—	—
		30 " " "	—	587	1,050	1.7	337
		60 " " "	—	417	721	1.7	167
J.R.P. (L) 18.3.25	210 steps per min.  Fore period : 2 mins.	30 secs. during expt.	45.6	1,764	1,980	1.1	1,514
		20 " " recovery	43.8	1,423	1,625	1.1	1,173
		20 " " "	33.4	1,132	1,320	1.1	882
		20 " " "	34.0	1,003	1,285	1.3	753
		30 " " "	28.7	588	993	1.6	338
		30 " " "	25.3	455	784	1.7	205
		60 " " "	20.3	328	587	1.7	78
J.R.P. (E) 19.2.25	188 steps per min.  Fore period : 60 secs.	30 secs. during expt.	40.8	1,734	1,890	1.0	1,484
		20 " " recovery	37.2	1,302	1,495	1.1	1,052
		20 " " "	35.0	1,218	1,515	1.2	968
		20 " " "	33.6	913	1,380	1.5	663
		30 " " "	30.8	656	1,170	1.7	406
		30 " " "	—	518	855	1.6	268
		60 " " "	—	441	760	1.5	191

In Table II are given values of the pulse rates in beats per minute, calculated from the times of successive groups of 10 and 20 beats. The figures above the horizontal line are for the period just before the exercise was stopped. Table II refers only to four experiments of Table I. The results of these four experiments are shown graphically in fig. 1.

Table II.

Subject K.F. (I)		Subject R.M.S. (H)		Subject: R.J.L. (I).		Subject J.R.P. (L)	
Time before or after end of exercise.	Heart rate in beats, per min	Time before or after end of exercise	Heart rate in beats, per min	Time before or after end of exercise	Heart rate in beats, per min	Time before or after end of exercise	Heart rate in beats, per min
secs		secs.		secs		secs	
— 8 7	169 5	- 12 4	178	7 6	184	12 6	165 1
- 5 2	169 8	— 5 5	175 3	- 4 5	184	— 9	166
- 1 7	170	- 1 7	176	3	183	— 5 4	166 5
			177	1 5	184	— 1 7	166 3
1 7	168 5	1 7	175	1 5	182 5	1 7	166 8
5 3	165	5 2	174	4 8	183 5	5 3	167
8 9	159 7	8 8	172	8 1	183	8 9	165
12 8	156 7	12 6	167 5	11 4	181 5	12 6	164 5
16 6	154 1	16 2	164 5	14 6	180	16 0	161 7
20 6	152	19 7	162	18	180 5	20 0	160 8
24 6	148	23 6	160	21 4	178 8	23 8	160
28 6	142 8	27 8	155 8	25	177	27 7	155 8
33 0	139 9	33	154 8	28 3	174 5	31 6	154 5
37 3	140	37	156	31	171 5	35 5	152 8
41 6	137	44	152	35	168 5	39 5	149 5
46	130	52	148	38	166 5	43 5	147 5
50 8	128	60	145	67	158	52 1	141 5
55 4	126 7	68	143 5	75	155 5	60 8	135
67	125 8	77	143	83	152	67	133 5
87	118 4	85	141	99	146 5	76	130 5
97	116 4	96	140 5	125	140 5	85	127 5
107	113 7	108	140	140	139	95	125 8
118	112 7	—	—	—	—	100	125
175	103 2	187	129 5	200	126 5	175	113 5

The heart rates in the other six experiments are given in Table III. These records also were measured for 10-beat intervals as in Table II; when the combined results are plotted, the curves are similar to those shown in fig. 1.

Four typical curves are shown in fig. 1. In each case the normal resting value, both of the oxygen intake and of the heart rate, was subtracted from the found value, and the figures so obtained were reduced to a scale of 100, in order to make easy the comparison of oxygen intake and pulse rate.

Table III.

Subject	R.M.S. (G).	R.M.S. (K).	R.J.L. (D).	K.F. (F).	J.R.P. (B).	J.R.P. (E).
Time.	Heart beats per minute.					
End of exercise	185	179.8	179.2	181.5	182	164.3
2 secs. after end of exercise	185	179.6	180.6	181	—	164.8
10 secs. " "	177	169	174	175	178	163
30 secs. " "	168	157	163	160	172	155
50 secs. " "	163	151	153	146	165	140
75 secs. " "	159	152	142	134	158	123
105 secs. " "	155	149	131	123	150	117
150 secs. " "	145	141	120	107	—	110

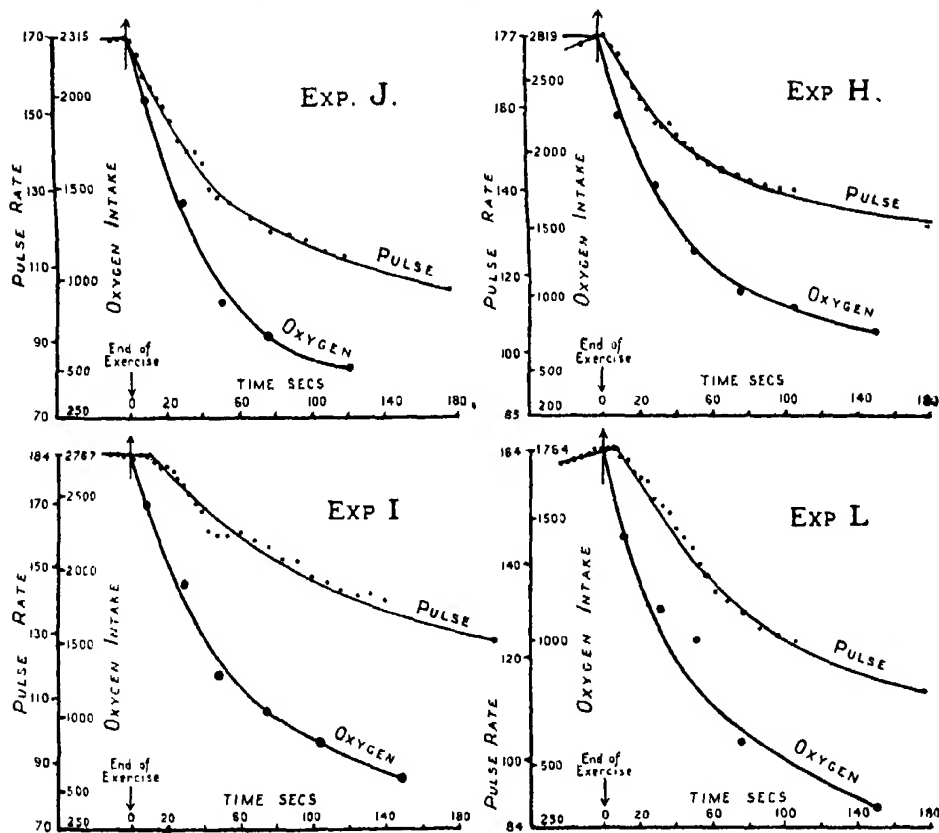


FIG. 1.—Horizontally, time in seconds from the end of exercise. Vertically, pulse rate and oxygen intake measured from the resting level and calculated as a fraction of the maximum rise (observed during exercise). Absolute values of the oxygen intake (cubic centimetres per minute), and of the heart rate (beats per minute), are marked along the vertical axis.

*Discussion.*

It can be seen from the curves of fig. 1 that both the oxygen consumption and heart rate fall off after the end of exercise in a similar manner, viz, rapidly at first and then slower, and that the rate of fall is roughly inversely proportional to the time. The fall of the oxygen-intake curve is the more rapid and it reaches the basal value sooner than the heart rate. A heart rate appreciably greater than normal was maintained for anything up to half an hour in our experiments; it does not serve any obvious function; it may not occur in highly trained individuals.

The oxygen intake reckoned per beat of the heart is given in Table IV

Table IV.—Oxygen Intake in Cubic Centimetres per Beat for Each Experiment.

	K	H.	D.	I.	E.	L.	B	I.	F
End of exercise	16.8	15.9	14.3	15.1	10.65	10.35	10.0	13.6	13.7
10 secs. after end of exercise	13.9	13.5	12.8	13.2	8.0	8.6	8.6	12.3	12.5
30 secs.       "       "	11.4	11.2	10.0	10.95	7.9	7.25	7.35	9.95	10.7
50 secs.       "       "	10.8	8.9	7.75	7.85	6.5	7.0	5.9	6.8	9.15
75 secs.       "       "	7.8	7.25	5.9	6.55	5.25	4.5	—	5.7	6.0
105 secs.       "       "	7.65	6.4	3.9	5.6	4.4	3.7	3.9	—	5.45
150 secs.       "       "	6.05	5.35	3.75	4.4	4.00	2.8	—	—	4.9
At rest	3.0	3.0	3.6	3.6	3.0	3.0	3.0	3.6	3.6

The results of these nine experiments are in good agreement and the mean values, which are as follows, are plotted in fig. 2.

*Mean Values.*

During exercise	.....	13.4 c.c. per beat.
10 secs. after exercise	..	11.5       "
30       "	..	9.6       "
50       "	..	7.8       "
75       "	..	6.1       "
105       "	..	5.1       "
150       "	..	4.5       "
∞       "	..	3.3       "

As recovery progresses there is clearly a marked falling off in the intake of oxygen reckoned per heart beat. This might be due to any or all of three factors: (1) To a greater saturation of the arterial blood during the exertion;

(2) to a greater coefficient of utilisation of the oxygen in the blood during exertion; and (3) to a greater output of blood per beat during exercise. Of these factors the first probably works in the opposite direction; during *severe* exertion, when the circulation rate of the blood is high, the arterial blood is almost certainly *less* well saturated than it is when circulating slower, as in recovery or at rest. The second factor is certainly one of the causes of the results given in fig. 2. Lindhard (11) has shown that there is a large

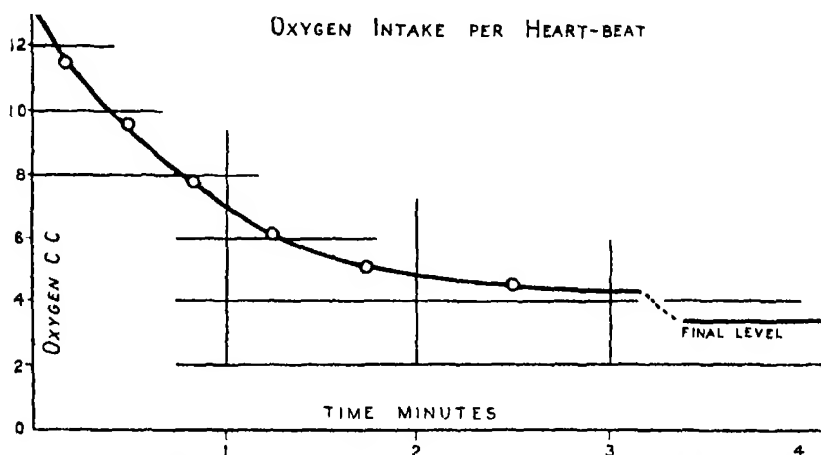


FIG. 2.—Oxygen intake during early stages of recovery from severe exercise.—Mean of nine experiments on four subjects—Oxygen in cubic centimetres per heart beat, time in minutes from end of exercise.

increase in the coefficient of utilisation of the oxygen in the blood, resulting from hard muscular work. A utilisation coefficient of about 30 per cent. at rest attained a value in one case as high as 80 per cent. during work. Similar large increases have been found by other workers, especially in some types of exercise [ (see Liljestrand and Collett (12) ) ]. This effect, however, does not appear to be large enough fully to explain our observations. The call for oxygen by the tissues is not appreciably altered immediately after the end of exercise from what it was during exercise. If the pulse rate were to fall off while the output per beat remained constant, then the oxygen intake would fall off at the same speed as the pulse rate. In addition to this, however, the slower circulation of the blood through the muscles would tend towards a higher coefficient of utilisation, and a greater intake of oxygen per litre of blood flowing through the lungs. We should expect, therefore, that the oxygen intake curve would fall off less rapidly than the heart-rate curve if the output per beat remained constant. This it does not do. Moreover, in some subjects the

oxygen intake can be increased more than 20 times (at any rate, while breathing rich oxygen mixtures), with only a three-fold increase in the pulse rate; it is difficult, on the published evidence available, to imagine a seven-fold increase in the coefficient of utilisation during exercise. Even in our experiments the effect would appear not to be large enough to be the only cause of the phenomenon shown in fig. 2.

Assuming that at rest the arterial blood of our subjects was 95 per cent saturated, and the mixed venous blood 65 per cent. saturated, both very reasonable assumptions [see, however, Henderson (13), who maintains that the coefficient of utilisation at rest is much less than is commonly recorded], we may calculate (on the assumption that the output of the heart per beat remains constant) that the maximum oxygen intake per beat would be  $95/30 \times 3.3$ , that is 10 c.c. This involves the impossible assumption that the mixed venous blood may, during exercise or in the early stages of recovery, be completely unsaturated. Even with this assumption the number calculated is only three-quarters of the maximum actually found, so it is obvious that a better utilisation during exercise of the oxygen in the blood cannot explain the phenomena. There would appear therefore to be little possibility of avoiding a resort to the third factor to explain our results, namely, an immediate decrease in the output of the heart per beat when exercise ends.

According to Bainbridge (5) a change in the total output of blood from the heart per minute may be caused by a change either in the output per beat or in the heart rate. At the end of exercise the pumping action of the skeletal muscles on the blood ceases abruptly, and if the pulse rate remained constant there would be a smaller filling of the heart during diastole, so that the output of the heart per beat would be largely diminished. This effect on his view would be to some degree antagonised by the withdrawal of the so-called "Bainbridge reflex," the slower filling of the heart during diastole leading to a fall in the pulse rate. It will be seen from our figures that the heart rate does not in all cases commence to fall immediately after the end of exercise, but in some remains up for a few seconds. The apparent rise in a few experiments is probably due to error. The venous pressure may well be maintained for a few seconds after the end of exercise, and consequently the heart rate remain at the same level. Bainbridge's view, therefore, would adequately explain our observations.

According to Boothby (7) the increased output of the heart per minute is determined almost entirely by the increase in the pulse rate. He found that not only the output of the heart per minute but also the heart rate have a



linear relation to the oxygen consumption. Douglas and Haldane (6) found that the percentage utilisation of the oxygen in the blood rises from 20 per cent., the resting value, to about 65 per cent. for the moderately severe exercise taken by their subjects. These values might be just sufficient to explain our results, on the assumption that the output per beat of the heart is invariable. The percentage utilisation of oxygen calculated from the figures given by Lindhard (8) lies between 60 and 65 per cent. during exercise and is about 30 per cent. during rest. If these values be used we have necessarily to assume an increase in the heart's output per beat during exercise. Douglas and Haldane showed that in the type of exercise employed by them, namely, working on a bicycle ergometer, the blood output per heart beat is in many subjects no greater during moderate or hard work than during rest. In other subjects, however, they admit that the output per beat is considerably less during rest and increases considerably during work. The important difference between their experiments and ours lies in the type of exercise performed. During standing running the body is free and the movements are rapid: in working on a bicycle ergometer the body is restrained and the movements slow. The venous return of blood in our case would be relatively easy as compared with theirs. Consequently, if heart output per beat depends upon rate of venous inflow, it is experiments on such forms of exercise as standing running which should show the greatest output of the heart. A divergence between our conclusions and those of other investigators might well be due to differences in the type and severity of exercise performed, especially in relation to the venous return of blood to the heart during diastole.

One further piece of evidence is found in the experiments recorded here, of a sudden change in the circulation rate of the blood when exercise ends. The respiratory quotient *during* exercise, as recorded in Table I, is never very high. About 20 secs. after the termination of exercise it begins to rise rapidly, often to very high values. This is not due to an increased ventilation of the lungs. If the blood suddenly starts to run more slowly, the amount of oxygen taken in will fall off roughly in proportion, the amount, however, of  $\text{CO}_2$  given out will not fall off in proportion, since it is not limited, as is the amount of oxygen taken in, by the physical circumstances of its carriage in the blood; a sufficient ventilation can wash large amounts of  $\text{CO}_2$  out of a small amount of blood. If, therefore, the circulation rate fell off rapidly when exercise ended, without any corresponding fall in the lung ventilation, we should expect to find, as we actually do, that the  $\text{CO}_2$  output would not fall off as rapidly as the oxygen intake, so that the respiratory quotient would rise.

*Summary.*

1. Determinations have been made of the oxygen consumption and of the heart rate during severe exercise (standing running) and during the early phases of recovery. The fall in oxygen consumption and the fall in heart rate commence abruptly, or almost abruptly, when bodily movements cease. The fall in oxygen consumption, however, is considerably more rapid than that in heart rate; it is due presumably to a sudden decrease in the circulation rate of the blood.

2. The oxygen intake *per heart beat* falls immediately and rapidly when exercise ends, even at a time when there is still a considerable demand for oxygen by the tissues. It would appear unlikely that the coefficient of utilisation of the oxygen in the blood should fall sufficiently to account for this, at a time when the circulation rate has diminished without any corresponding diminution in the demand for oxygen. An increased coefficient of utilisation certainly occurs during severe exercise, but it must be accompanied by an increased output per beat, which returns rapidly to its usual value on the cessation of movement.

3. It is suggested that the freedom and rapidity of the movements performed by the subjects of our experiments are the fundamental difference between the observations recorded here, and those in which the output of the heart per beat, as measured by other observers, has remained the same during exercise and at rest.

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*Muscular Exercise, Lactic Acid, and the Supply and Utilisation of Oxygen.—Part XII. A Note on the Technique of Determining the Resting Oxygen Intake while Breathing Concentrated Oxygen Mixtures.*

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In a recent paper Hill, Long and Lupton (1) discussed the employment of the Douglas bag technique for determining the oxygen consumption while breathing strong mixtures of oxygen. They found experimentally that the apparent oxygen intake at rest might differ appreciably from that found while breathing air, and concluded that this apparent difference was caused by an error in the technique due to the assumption (not strictly true, except after very long periods of breathing the gas mixture) that the amount of nitrogen in the expired air is precisely equal to that in the inspired air.

It was conceivable, of course, that the difference observed was a genuine one and due to an increased usage of oxygen at a higher oxygen pressure. This was unlikely in view of the fact that the subjects were healthy men at rest, their oxygen supply being certainly in no sense inadequate while breathing air, other reasons, moreover, were given against this possibility.

Pflüger in 1893 (2) had already stated that the respiratory exchanges are determined by the needs of the cells and not by the oxygen supply. Later on Benedict and Higgins (3) confirmed this view, observing that the oxygen absorbed is the same whether the breathing mixture contains oxygen below or above its normal pressure. In their experiments they used a modification of the closed space apparatus described by Benedict (4). Krogh (5), however, criticised the results obtained by these investigators, and affirmed that the increase in the oxygen pressure does produce an increase in the rate of oxidation. A similar conclusion was arrived at by L. Hill and Flack (6), who found that breathing oxygen increases the rate of oxygen consumption during the periods of muscular exercise and recovery. Briggs (7) pointed in the same direction, although he restricted his conclusions to untrained men. During fatiguing exertion, however, he observed a beneficial effect of oxygen breathing,

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no matter how fit the men were. His technique appears to have been quite deficient, for—apart from the impossibility of employing the Douglas bag method with any accuracy at very high oxygen percentages—he did not attach any importance to the period of collection of the samples of expired air [see Lupton (8)], and when he attempted to avoid the inherent inaccuracy of the method he measured the amounts of the inspired and of the expired air with two *different* jerky gas-meters, one of which, as he confessed, was not very accurate. Hill, Long and Lupton (9) have recently shown that the time of recovery is not shorter when oxygen-enriched air is breathed. A more decisive and direct test, therefore, seemed to be required, and this has been furnished by the following investigation.

The resting oxygen intake was determined after a suitable fore-period of rest, using both the Douglas bag technique, breathing air, and the Krogh's respiration apparatus (10) filled with cylinder oxygen (95.98 per cent.) Table I shows the results obtained. The average difference between the oxygen intake values, breathing air and oxygen, is only 3.4 c.c., the Douglas bag technique showing in this series slightly the larger value.

Table I.

Subject	Basal value (Douglas) ( $\text{O}_2/\text{O}_2$ )	Basal value (Krogh) $\text{O}_2$	Date, 1924
J R P	205/239 c.c. per min.	200	Oct. 7.
J R P	182/225 ..	224	Oct 10
J R P	184/233 ..	220	Oct 13.
J R P	205/230 ..	230	Oct 22
J L P	224/275 ..	276	Oct 23
J R P	210/237 ..	225	Nov 10
K F	215/268 ..	267	Nov 11
K.F.	209/245 ..	246	Nov. 13
K F.	174/238 ..	270	Nov 13
K F.	215/268 ..	260	Nov 14.
J.R P	275/279 ..	257	Nov. 16.
K F.	190/235 ..	236	Nov 19.
J.R P.	219/260 ..	268	Nov 21

It is clear, therefore, that the breathing even of nearly pure oxygen gives no greater metabolism at rest than breathing air. Evidently the difference found by Hill, Long and Lupton lies—as they thought—in an error due to the Douglas bag technique when employed with strong oxygen mixtures under the conditions described.

That this is so is shown by further experiments in which the oxygen consumption was determined after a suitable fore-period of rest :—

(a) by the Douglas bag technique, breathing air ;

(b) by the Douglas bag technique, breathing a rich oxygen mixture , and

(c) by a modified Douglas bag technique in which the volumes of the inspired and expired air were separately measured, no assumption being made as to the constancy of the nitrogen. Special precautions were taken in measuring these volumes. The subject lay comfortably on a couch and breathed the oxygen mixture, which passed smoothly through a water gas-meter. The expired air was collected in a bag, previously washed out with the subject's own expired air. After such collection, a small portion of the air from the bag was taken in a sampling tube and the remaining air measured with the *same* gas-meter. The known amount of the air in the sampling tube was then added to the calculation.

When strong oxygen mixtures were used, the analysis of the expired air was made by means of the Haldane gas-analysis apparatus as modified by D. T. Harris \* Typical results are shown in Tables II and III. It appears clearly that the result of method (c) agrees closely with that of method (a), but is appreciably less than that of (b).

Table II.—Comparison of methods (a) and (b).

November 7, 1924	Subject, K.F.	Temp., 16° C.
Basal value, breathing air, lying on a couch —		
Foreperiod of rest		20 min.
Ventil. per min.		5.3 lts.
Respiratory quotient, 0.84 (228/272)		
Breathing mixture (O <sub>2</sub> , 44.32 per cent) —		
Time of collection		0 to 10 min.
Ventil. per min.		5.1 lts.
Respiratory quotient, 0.51 (223/436)		
Time of collection		10 to 20 min.
Ventil. per min.		5.08 lts.
Respiratory quotient, 0.76 (230/301)		
Time of collection		20 to 30 min.
Ventil. per min.		5.3 lts.
Respiratory quotient, 0.87 (228/262)		
Returning to air. —		
Time of collection		30 to 40 min.
Ventil. per min.		4.97 lts.
Respiratory quotient, 1.53 (212/139).		
Time of collection		40 to 50 min.
Ventil. per min.		4.43 lts.
Respiratory quotient, 0.82 (184/222).		
Time of collection		50 to 60 min.
Ventil. per min.		5.21 lts.
Respiratory quotient, 0.87 (228/263).		

\* Made by Band & Tatlock, London.

Evidently here the variation in the oxygen intake is due to a false assumption in (b), viz., that the nitrogen is constant.

Table III.—Comparison of methods (a), (b) and (c)

December 24, 1924 Subject, J.R.P. Temp, 17° C.		
Basal value, breathing air, lying on a couch —		
Foreperiod of rest		20 min.
Time of collection		10 min.
Ventil. per min.		5 15 lits.
Respiratory quotient, 0.93 (225/241)		
Breathing mixture (O <sub>2</sub> , 40.2 per cent) —		
Foreperiod		5 min.
Time of collection		10 min.
Ventil. per min.	{ Inspired air Expired air	4.675 lits.
		4.664 lits.
Respiratory quotient	Direct calculation, method (c)	0.89 (215/242).
	Indirect calculation, method (b)	
	(N <sub>2</sub> assumed to be constant)	0.82 (215/262).

There is no ground, therefore, for the belief that mixtures containing a high percentage of oxygen increase the rate of oxidation at rest.

My thanks are due to Prof. A. V. Hill, who suggested the work here reported, and to Mr. K. Furusawa, who assisted me in the conduct of the experiments.

### *Summary.*

1. The oxygen intakes at rest (a) while breathing air, the expired air being analysed with the Haldane's gas-analysis apparatus, and (b) while breathing nearly pure oxygen (95–98 per cent.), using in this case the Krogh's apparatus, are practically the same.

2. The greater oxygen consumption found at rest while breathing rich oxygen mixtures, the expired air being analysed with the Haldane's gas-analysis apparatus, is due to an error occurring in this case in the Douglas bag technique, viz., the incorrect assumption that the total amount of nitrogen is the same in the inspired as in the expired air. If the amounts of the inspired as well as of the expired air be measured separately by the same meter, no assumption being made as to the constancy of the nitrogen, the results obtained as to the oxygen used are identical, whether breathing air or strong oxygen mixtures.

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*The Inhibitory Effect of Blood Serum on Hæmolysis.*—II.

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In a paper previously published (1), in which the inhibitory effect of serum on the hæmolysis produced by saponin and the bile salts was subjected to a detailed quantitative examination, certain points were left untouched, as requiring further investigation. It was therein shown that, if the non-hæmolytic substance which results from the interaction of the serum and the saponin or bile salt is regarded as playing no part in the hæmolytic reaction after its formation, there is a simple relation between the amount of hæmolytic substance acting on the cells and the amount rendered inactive by the inhibitory substance, so that, if the former amount be indicated by  $c_2$  and the latter amount by  $x$ , the simple expression

$$x = Ac_2^{1/n}$$

describes the relation of the two quantities,  $A$  and  $n$  being two constants. It was further found that these constants varied with many circumstances, and principally with the dilution of the serum employed for the purpose of bringing about inhibition; with this variation the present paper is concerned. It may be noted that the validity of the formula has been confirmed by Kennedy (2), who has found it to apply also to the inhibition produced by glucose and fructose on saponin hæmolysis.

In this paper we shall extend the results of (1) and deal with the value of the constants  $A$  and  $n$  under various conditions.

*Methods.*

The methods employed are, in general, those of the above-mentioned paper, to which the reader is referred for detail. For the present purpose, saponin dissolved in saline (0·85 per cent. NaCl) in concentrations from 1 in 10,000 to 1 in 80,000, and sodium taurocholate in saline from 1 in 1,000 to 1 in 7,000, were used as the hæmolytic agents. The majority of the experiments, however, were carried out with the saponin, since the instability of the bile salts and their tendency to spontaneous alterations of hæmolytic activity are so marked and so impossible to control. These hæmolytic solutions were made up as soon as possible before the performance of the actual experiment.

The cell suspension was one prepared by suspending the washed cells from 1 c.c. of human blood in 20 c.c. of saline—the standard suspension usually employed by the writer. The sera used in the experiments were obtained from the clotting of blood from the animals concerned, and were employed suitably diluted with saline.

The great length of some of the experiments made it desirable that the whole of certain parts of the investigation should be conducted on one sample of serum; for this purpose a sterile horse-serum was used, a small quantity being withdrawn from the sealed container every day. No variation occurred in the inhibitory power of this specimen over a period of three months; results obtained from different experiments in which it was used may therefore be considered comparable.

*The Value of the Constants with Various Dilutions of Serum.*

In the paper referred to above, it was found that there are very considerable variations in the values for the constants  $A$  and  $n$ , when the same amount of serum is used for producing inhibition. Thus, with the employment of 0·01 c.c. of human serum, there were obtained values of  $A$  from 0·28 to 0·455, in the case of saponin hæmolysis, and of  $n$  from 1·18 to 2·66, similarly, in the case of taurocholate hæmolysis in the presence of 0·01 c.c. of human serum, values of  $A$  from 0·5 to 0·894, and of  $n$  from 1·88 to 3·3. These figures indicate plainly enough that two samples of human serum may produce very different inhibitory effects, even if present in the same amount. It was also noted that the value of the constants depended, in the case of any one serum, on the amount of the serum present as an inhibitory substance. It might be expected that if a certain quantity of serum inhibited a certain amount of hæmolytic agent, that twice the amount of serum would inhibit twice the amount of hæmolytic substance, but this is far from being the case. In general, the amount of



hæmolytic agent inhibited per unit of serum becomes less as the quantity of serum is increased, and variations in the value of the constants  $A$  and  $n$  accordingly appear to take place as the amount of inhibitory substance is greater or smaller.

These quantitative relations have therefore been studied in greater detail, by finding the time-dilution curves for saponin acting on cell suspension in the presence of various quantities of serum. The investigation suffers from certain experimental limitations, for it is impossible to obtain satisfactory curves with greater quantities of serum than about 0.04 c.c., or with less quantities than about 0.00125 c.c., when the dilutions of saponin such as are commonly employed are used. Nor is it practicable to extend the experimental range by using more concentrated solutions of saponin than the customary 1 in 10,000, or less concentrated ones than about 1 in 80,000 (at 25° C.). Over this range, however, the phenomena can be observed without any difficulty.

The matter is most clearly presented by giving one experiment in detail, and following it by a discussion, and by a brief summary of a number of other experiments

*Experiment 1.*—Saponin and horse serum at 25° C. Standard curve, saponin in absence of serum, passes through the following points: (10,000, 0.25), (20,000, 0.7), (25,000, 1.0), (30,000, 1.5), (35,000, 2.0), (40,000, 2.4), (60,000, 6.5), (70,000, 13).

	$\delta_1$	$c_1$	T.	$\delta_2$	$c_2$	$x$
Saponin plus 0.04 c.c. serum	10,000 13,000 15,000	0.200 0.154 0.133	1.8 5.0 12.0	33,000 55,000 69,000	0.061 0.036 0.285	0.139 0.118 0.104
Saponin plus 0.02 c.c. serum	10,000 15,000 20,000 25,000	0.200 0.133 0.100 0.080	0.7 2.1 5.0 13.0	20,000 36,000 55,000 70,000	0.100 0.550 0.036 0.028	0.100 0.078 0.064 0.052
Saponin plus 0.016 c.c. serum	15,000 20,000 25,000 30,000	0.133 0.100 0.080 0.066	1.5 3.2 6.3 16.0	30,000 44,000 59,000 71,000	0.066 0.045 0.034 0.028	0.066 0.055 0.046 0.038
Saponin plus 0.01 c.c. serum	20,000 25,000 30,000 35,000	0.100 0.080 0.066 0.057	2.0 3.2 5.5 10.0	35,000 45,000 57,000 66,000	0.057 0.044 0.035 0.030	0.043 0.036 0.031 0.027
Saponin plus 0.0083 c.c. serum	20,000 25,000 30,000 35,000	0.100 0.080 0.066 0.057	1.4 2.4 3.8 5.5	32,000 40,000 49,000 56,000	0.062 0.050 0.040 0.035	0.038 0.050 0.026 0.022

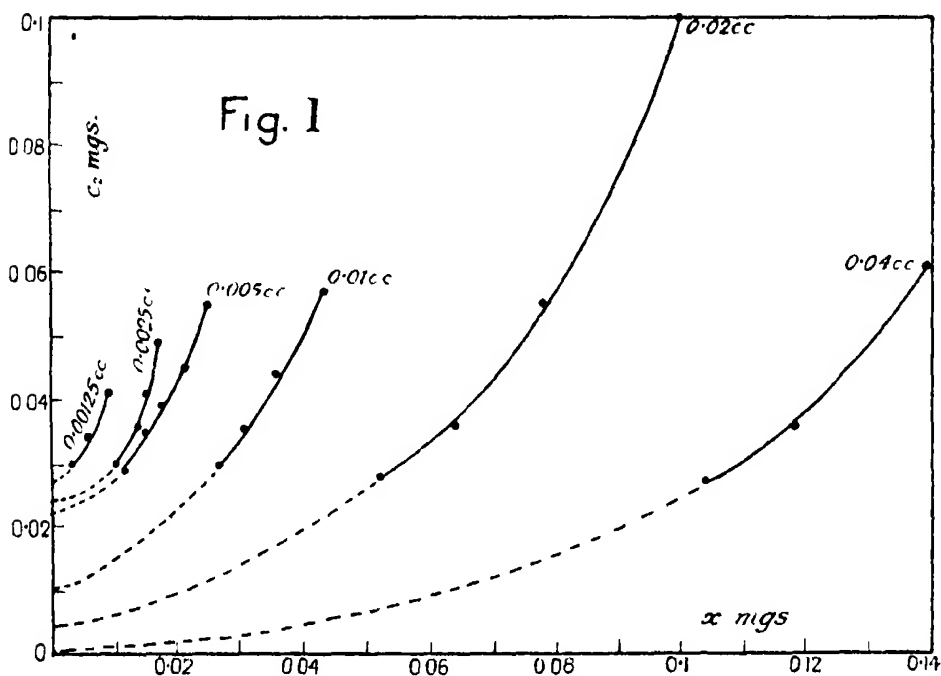
## Experiment 1.—(contd.)

	$\delta_1$	$c_1$	T	$\delta_2$	$c_2$	$x$
Saponin plus 0.005 c.c. serum	25,000	0.080	2.1	36,000	0.055	0.025
	30,000	0.066	3.0	44,000	0.045	0.021
	35,000	0.057	4.0	51,000	0.039	0.018
	40,000	0.050	5.2	56,000	0.035	0.015
	50,000	0.040	11.0	67,000	0.029	0.011
Saponin plus 0.00426 c.c. serum	30,000	0.066	2.4	41,000	0.048	0.018
	35,000	0.057	3.5	46,000	0.043	0.014
	40,000	0.050	4.0	52,000	0.038	0.012
	50,000	0.040	10.0	66,000	0.030	0.010
Saponin plus 0.0025 c.c. serum	30,000	0.066	2.5	41,000	0.049	0.017
	35,000	0.057	3.5	46,000	0.049	0.016
	40,000	0.050	5.0	55,000	0.036	0.014
	50,000	0.040	10.0	66,000	0.030	0.010
Saponin plus 0.00125 c.c. serum	40,000	0.050	3.5	48,000	0.041	0.009
	50,000	0.040	6.0	58,000	0.034	0.006
	60,000	0.033	10.0	66,000	0.030	0.003

If, in the case of each dilution, we plot  $\log c_2$  against  $\log x$ , a good straight line results, but the slope of the different lines varies greatly, being different for each dilution of serum. This means that there is a different value of  $n$  for each dilution of serum, and also a different value of  $A$ ,  $n$  varying in this experiment from about 0.35 to about 3.5, and  $A$  varying between about 0.3 and 90.0. It will further be seen that the lines cut each other; an impossible state of affairs.

In order to simplify the matter, we may plot, not  $\log c_2$  against  $\log x$ , but  $c_2$  against  $x$  itself, both quantities being expressed, as before, in milligrammes. The result of doing this is shown in fig. 1, where the results for only six out of the nine quantities of inhibitory agent are given, in order to avoid congestion of the figure. We have here a series of curves, suggestive of parts of parabola, which clearly cut the  $c_2$ -axis at different levels. When a considerable amount of serum is present (0.04 c.c.) the curve passes very nearly through the origin; when small quantities of serum are used (0.00125 c.c.), the curve appears to cut the  $c_2$ -axis at a point considerably above the origin. Owing to the limited experimental range, it is not possible to say exactly where each curve cuts, but, going on the assumption that each is a part of a parabola, the point of section can be determined with sufficient accuracy, and the whole curve drawn in.

Now, selecting any curve, and calling the co-ordinates of the point where it cuts the  $c_2$ -axis, O, K, we can plot  $\log (c_2 - K)$  against  $\log x$ ; such a procedure



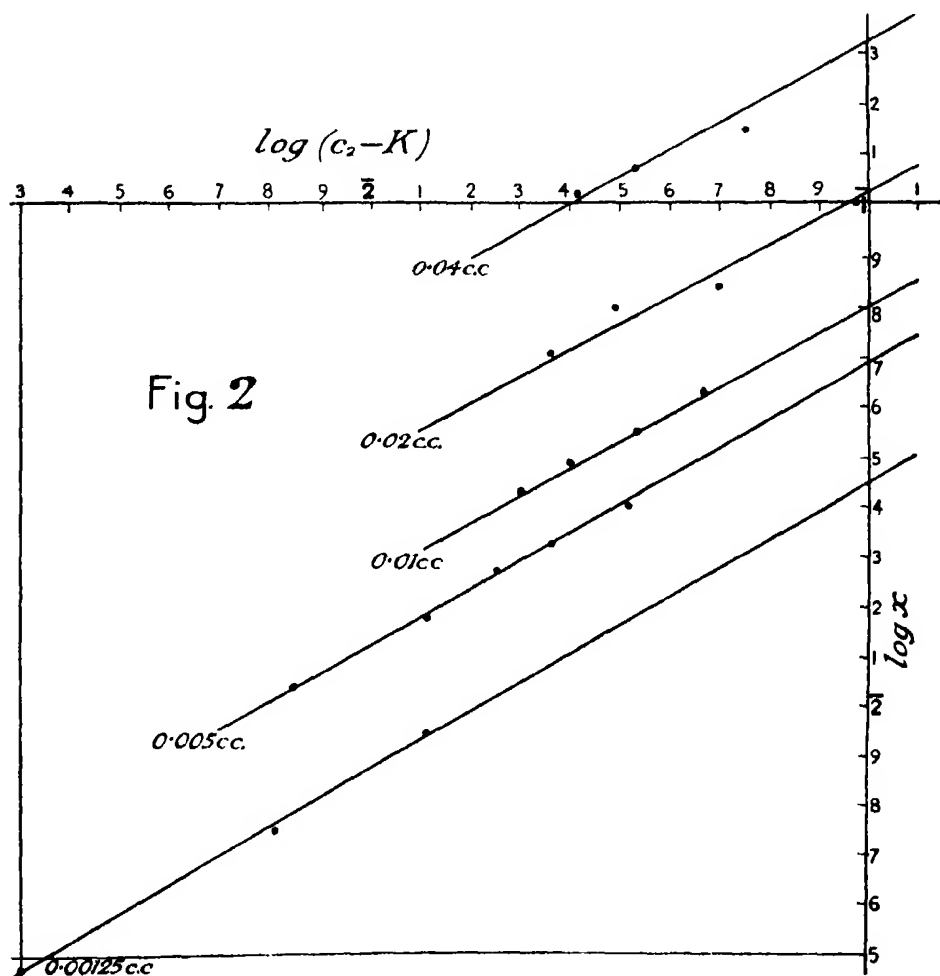
results, for each curve, in a straight line. These lines are shown in fig. 2, the following values being taken for K :---

Serum.	Value of K.	Serum.	Value of K.
0.04	0.003	0.005	0.022
0.02	0.005	0.00125	0.028
0.01	0.010		

The lines resulting from this manner of plotting are parallel, with a slope of  $n = 1.75$ . The points lie on them very well, although not perfectly, but the important matter is that the taking into account the constant K has simplified the whole problem. If this constant be ignored, and  $\log c_2$  be plotted against  $\log x$ , the result must be of necessity a complex series of curves, which, owing to the experimental range being so restricted, may be mistaken for a series of straight lines of different slope.

The lines shown in fig. 2 might well be the result of an adsorption experiment, in which serum was adsorbing saponin, according to the usual equation,

$$x = Ac_2^{1/n}.$$



We may now investigate the values of the constant  $A$ , which for these lines are as follows :—

Serum.	Value of $A$ .	Serum.	Value of $A$ .
0.04	0.74	0.005	0.16
0.02	0.36	0.00125	0.10
0.01	0.20		

For fairly large quantities of serum, twice the quantity of serum removes about twice the quantity of saponin from the same concentration; that is, if  $m$  is the amount of serum, the equation

$$x/m = Ac_2^{1/n}$$

the usual adsorption equation for varying amounts of adsorbing agent—appears to hold. When the quantity of serum becomes very small, this expression is departed from somewhat, small amounts of serum being able to dispose of somewhat greater amounts of saponin than would be expected on theory.

Considering the complex nature of the experiments, the closeness of the results to those which might be expected on the assumption that the serum forms an adsorption compound with the saponin is striking. This must not, of course, be taken as proof that an adsorption process is responsible for the phenomena, but it may be taken to show that there is nothing inadequate about such an explanation, which fits the facts better than any other at present known. The similarity between the two processes would, indeed, be complete were it not for the appearance of the constant  $K$ , which regulates the origin of the parabolæ. Put into words, the meaning of its appearance is that when any particular quantity of serum is used as an inhibitory agent, some of the hæmolytic agent which acts on the cells is not available to enter into the adsorption reaction between the serum and the hæmolytic agent. The amount of this non-available hæmolytic agent varies with the amount of the inhibitory agent added, when the latter is great, the former is small. Considering the nature of the reaction under observation, this is not a surprising finding, and it is tempting to show that it is exactly what might be expected. The accuracy with which the values of  $K$  can be determined is, however, not great enough to warrant this being done in the meantime.

The results of this representative experiment having been discussed in full, we may record the results of others similar. In each case the procedure was the same; the proper values of  $K$  were first determined, and thereafter the values of  $A$  and  $n$ . The values of the latter constant are shown below, for a series of 10 experiments.

Experiment	Value of $n$	Experiment	Value of $n$ .
1	2.0	7	2.0
2	2.3	8	1.8
3	1.8	9	1.9
4	1.7	10	2.1
5	1.9		
6	2.1	Mean	1.9

These values are the best values for each experiment, and, on the whole, are remarkably constant, being all in the neighbourhood of 2.0. The values of  $A$  vary, in these experiments, according to the amount of inhibitory substance present, twice the amount of inhibitory substance giving about twice the value of  $A$ , except when the amount is very small.

All these experiments were conducted with serum as the inhibitory agent and saponin as the lysis. Certain experiments have also been carried out with sodium taurocholate as the hæmolysin; but these experiments, owing to the marked instability of the bile salt, are much less satisfactory. They demonstrate, however, that the reaction between serum and the bile salt is of the same general type as that between serum and saponin, and that the same methods which reduce the latter case to a simple form are applicable to the former. Inhibition experiments of a quantitative kind cannot be carried out satisfactorily with sodium glycocholate or with the soaps, the peculiarities which occur with the former, and the instability of the latter, being insuperable difficulties at present.

#### *The Effect of Temperature.*

If the reaction between serum and saponin is one involving phase-surfaces, it will be expected that it will be influenced by temperature. In order to see if this were so, experiments were carried out at various temperatures between 10° and 45°, the inhibitory effect of the same amount of the same serum being observed at each.

Over this necessarily restricted range no changes which could be considered significant were observed. Using the same amount of serum, say, 0.01 c.c., the same value of  $n$  was obtained whether the experiment was conducted at 10° or at 45°. In some experiments there were indications that, as the temperature increased,  $n$  became slightly smaller, but, as the changes were within the limits of possible experimental errors, no significance is attached to them.

In adsorption reactions in general, a rise of temperature diminished the amount of adsorption, although it increases its velocity, and with rise of temperature one would thus expect to find a diminishing value of both  $n$  and  $A$ . In these inhibition experiments, this was not observed, but the absence of the observation does not militate against the idea that the reaction is an adsorption process, for the temperature change is comparatively small.

*Conclusions.*

The results described in this paper, together with those of a previous paper (1) reduce the phenomenon of inhibition of certain hæmolytic substances by serum to order. As might be expected, the phenomenon is a complex one, for one is dealing with a system in which are present saponin, a semi-colloid, serum, a mixture of colloids, and cells, themselves complex in structure. When one considers the two quantities, the amount of hæmolysin inhibited by the serum, and the amount left unaffected, it is found that these quantities are related in such a way as to make it probable that the reaction between the lysin and the serum is an adsorption one. For if  $m$  is the amount of serum,  $x$  the amount of inhibited hæmolysin, and  $c_2$  the amount of lysin left unaffected, the usual adsorption isotherm,  $x/m = Ac_2^{1/n}$  applies within the limits of experiment, provided allowance is made for a certain variable amount of lysin which appears to act on the cells, but which is not available for entering the adsorption reaction.

It will be noted that the method of measurement applied to the phenomenon makes no attempt to find out how much lysin is inactivated by the serum before the addition of the cells, and therefore does not measure the equilibrium which results from the combination of the two surface-active colloids as a result of their mixture before the cells are added. It rather measures the quantities of free and inhibited lysin throughout the whole course of the hæmolysis of the cells—quantities which vary from time to time—and, while it proceeds on the assumption that the portion of hæmolytic agent inhibited by the serum is removed from the field of the reaction between the remaining lysin and the cells, it necessarily is not concerned as to whether this occurs or not. These points have to be borne in mind in the interpretation of the results; although these reduce to a simple and suggestive form, it is, strictly speaking, incorrect to regard the resulting formulæ as more than empirical, for the method is to some extent arbitrary, and does not take account of all the possibilities. The fact that the formulæ fit the experimental results so well renders them, however, valuable empirical formulæ, and their similarity to the well-known adsorption equation constitutes at least a point of interest.

*Summary.*

1. The results of experiments on inhibition of hæmolysis by serum, described in a previous paper, are amplified. It is shown that the reaction is described by equations similar to absorption equations, and that the inhibition is probably

due to the formation of a loose physical compound between the lysin and the serum proteins.

2. The special method necessary for the obtaining of the proper constants in the equations is illustrated and discussed.

3. It is shown that the reaction is little influenced by temperature within the experimental range

(This research was carried out during the tenure of a Crichton Scholarship in the University of Edinburgh, and the expenses defrayed by a grant from the Royal Society.)

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### *Fatigue and Plurisegmental Innervation of Individual Muscle Fibres.*

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(Communicated by Sir Charles Sherrington, P R S —Received June 23, 1925.)

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[PLATES 32, 33 AND 34.]

The classical experiments of Waller (1893) and others in which a muscle, fatigued to inexcitability by nerve stimulation, was shown still to respond to stimuli applied directly, led to the conclusion that fatigue occurred at the motor end-plate. Some have inferred from this and similar experiments that the hypothetical junctional tissue, when fatigued, conducts with a decrement (Lucas, 1917). These inferences have recently been supported by the experiments of Cattell and Stiles\* (1924), and independently by those of Beritoff (1924, *a* and *b*).

Though the usefulness of the theory of neuro-muscular fatigue commonly held has been very great, it seems, nevertheless, possible to place another interpretation upon Waller's, Lucas's, Cattell and Stiles's, and Beritoff's

\* A continuation of Cattell and Stiles's investigations by Shafer and Stiles (1925) has recently appeared, but this does not affect their previous conclusions.



experiments, and, as an alternative view is sometimes helpful in the solution of a complex problem, it is hoped that the following view may prove suggestive and of some value. A description will first be given of a repetition of Cattell and Stiles's and of Beritoff's experiments, using the optical method previously described (1925, a).\*

The gastrocnemius muscle in the frog *R. temporaria* is usually innervated by the VIIIth and IXth lumbar roots † In the majority of specimens, stimulation of either root causes well-marked contraction of the gastrocnemius muscle. This was probably first demonstrated by Panizza (1835) and Kronenberg (1836), and was subsequently investigated in greater detail by Eckhardt (1849) and by Peyer (1853). It is interesting that Panizza and Kronenberg regarded the dual nature of the innervation of many muscles as a protection against fatigue. Sherrington (1892) re-examined the problem, and has given a detailed historical account of the early investigations. He found, using intact muscle of a pithed frog, that fatiguing the gastrocnemius through one root had but little influence upon the response produced when the other root was subsequently stimulated. He noted, however, that in the absence of the circulation (p. 711), as after excision, the "test" root is more influenced by the "fatiguing" root, and he suggested that "fatigue" products tend in the absence of the circulation to diffuse more readily from fatigued fibres into unfatigued fibres.

He also drew attention to the fact that Krause and Eckhardt in their exhaustive search had failed to find in muscles innervated by more than one root that any individual fibre had more than one end-plate. Kühne (1886), however, found several rare instances of very long muscle fibres (frog) which possessed a motor end-plate at each end. Sherrington adds that Panizza and Kronenberg were broadly correct in supposing that a muscle fatigued through one root remained unfatigued when stimulated through the other, but he also

\* The present experiments were commenced before Cattell and Stiles or Beritoff's results were published, but they were originally directed toward the elucidation of another point, viz., the differences in duration of maximal and submaximal responses, the results of which are being published elsewhere (1925, d). Experiments on root stimulation of the gastrocnemius of a frog similar to those of Cattell and Stiles were performed in 1882 by Gad, who failed to find that the total tension developed when the roots were stimulated singly overlapped that developed when the roots were stimulated simultaneously. A similar result was obtained by Lederer and Lemberger (1907) on the *M. Cricothyroideus* of the rabbit, which supersedes the previous observations on this muscle by Exner (1884).

† Ecker and Wiedersheim (1898) give the roots of gastrocnemius (*R. esculenta*), as IX and X. In *R. temporaria*, however, roots VIII and IX are the predominant supply (Langley and Orbeli, 1910).

points out that peripheral fatigue pushed to inexcitability is unlikely in the intact animal, since central fatigue supervenes (Waller, 1891). It may be added that plurisegmental innervation of individual fibres is also unlikely on embryological grounds. Tello (1917) has studied with great care the outward growth of nerve fibres to their respective muscles, and his results suggest that once a nerve fibre has found its muscle fibre the latter is, as it were, "fertilized," and—as with twins—it seldom happens that a muscle fibre is twice "fertilized" by nerves. Also, if one supposed, for argument, that all individual muscle fibres have two end-plates, the laws of probability would be defied should more than 50 per cent be innervated plurisegmentally—that is, if the distribution of fibres in a distal appendage occurs at random, as indeed seems probable from the results of degeneration and regeneration (Sherrington, 1892).

Agduhr (1919), however, has described plurisegmental innervation\* of the individual muscle fibres of the rabbit. This has led Cattell and Stiles and Beritoff to consider the possibility of a similar condition holding in muscle fibres of the frog. Lindhard (1924) has reported plurisegmental innervation of fibres in the frog, but in view of Kuhne's most painstaking search for fibres showing two end-plates it is extremely doubtful whether a larger proportion of the muscle fibres are so innervated. If, however, as Cattell and Stiles rightly point out, a large proportion of individual fibres are (doubly) innervated by nerves from both roots, the muscle as a whole ought to respond, when stimulated through either root singly, with a vigour approaching that of the response when both roots are stimulated simultaneously. This point has in the present work been examined experimentally on the intact gastrocnemii of decerebrate frogs which were stimulated first through the VIIIth lumbar root, then through the IXth, and finally through both simultaneously. The mechanical response was recorded by a high frequency isometric torsion-wire myograph, and as a control to tension development the electrical responses have been taken simultaneously with the mechanical responses. Since alterations in the initial tension greatly alter the size of the mechanical response, care was taken to ensure that the initial tension was constant during any series of observations. Twelve experiments have been performed, and, as they have all given concordant results, I shall describe a typical experiment briefly.

The VIIIth root of a 35 gm. frog was stimulated at 50 per sec. for a short interval. The plateau tension developed was 500 gm. The IXth root when

\* In view of what follows it may be recalled that certain of Agduhr's histological findings have recently been called into question by Hunter (1925, p. 199), Kulchitsky (1924), and by Langley (1921).

stimulated developed 685 gm., giving a total of 1,185 gm. for the two singly (see Table I.). When both roots were stimulated together, 1,060 gm. were developed in the plateau. The sizes of the *plateau* electrical responses during these responses were respectively 4.5 mm. (VIII) : 5.0 (IX), a total of 9.5 mm. ; with VIII and IX together the responses were 8.9 mm. The photographic record of these responses is shown in fig 1 (*cf.* 2). This general degree of overlapping of the sum of the two responses taken singly and that of the responses taken simultaneously has appeared in the majority of observations (all but one, see fig. 2), but it amounts on an average to a difference of only about 10 to 15 per cent. both for the mechanical tension developed and usually for the size of electrical response. The electrical responses, however, tend to show rather less discrepancy, as Samojloff (1924) has recently observed, being in some observations as low as 4 or 5 per cent. In one record (fig. 2) no mechanical overlapping occurred, though there was 4 to 5 per cent. overlapping in the electrical responses.

Table I.—Stimulation of lumbar roots VIII and IX in a 35 gm. frog (*Rana temporaria*). Summary of three experiments. Tetanus composed of 50 break induction shocks per sec. T = 15.0° Muscle (gastrocnemius) intact.

Root	Twitch		Tetanus.	
	Electrical response.	Mechanical response	Electrical response.	Mechanical response.
VIII	mm	gm.	mm	gm
IX	7.8	105	4.8	600
	7.7	100	5.8	465
	15.5	205	10.6	1065
IX and VIII	15.0	135	10.0	985
	Tetanus		Tetanus	
	Electrical response.	Mechanical response	Electrical response.	Mechanical response.
VIII	9.0	400	4.5	500
IX	13.0	610	5.0	685
	22.0	1010	9.5	1185
VIII and IX	20.0	900	8.9	1060

In twitches the discrepancy between the mechanical tension developed, when stimulated in the same way through the roots, is much greater. In one experiment the sum of the two taken singly amounted to 205. When taken simultaneously the twitch plateau was reached at 135. The electrical responses, however, showed a discrepancy of only 3 per cent. This suggests that the mechanical tension developed is not necessarily an accurate index of the number of fibres participating in a reaction, even though initially they are under the same mechanical conditions of tension, etc. In Table I a series of observations is summarised.

The question, therefore, arises as to whether the discrepancy of 3 to 10 per cent. in the electrical responses is to be looked upon as evidence of plurisegmental innervation. It would seem to indicate that Cattell and Stiles' calculations of 85 per cent. of the fibres being so innervated is too high in estimate, at least if all frogs are the same in this regard. One might in fact quite reasonably ask whether this small discrepancy may not be accounted for in another way. An individual muscle fibre is pre-eminently an adaptive mechanism, and as Fenn (1923) and others have shown, it alters its energy output according to the amount of work it finds it must do. Is it not more probable that the 10 per cent. discrepancy is to be accounted for by the fact that when only half the fibres are contracting they individually and collectively develop more tension and liberate more energy than when they are assisted by the other portion of the fibres innervated by another root? This possibility is supported by the facts brought out in fig. 3 of another paper (Fulton, 1925, *d*), which show that the smaller the tendon shortening permitted the greater the tension developed (and the greater the size of the 'plateau' electrical responses). When only a portion of the fibres respond they shorten less than when assisted by the remaining portion. Moreover the terminal mechanical response of a tetanus ('after-action') becomes more prolonged as the extent of shortening permitted is made less. One would, therefore, anticipate that the after-action of the response when one root is stimulated singly would be greater than when both roots are stimulated together. An examination of figs. 1 and 2 proves that this is clearly the case. Thus, in fig. 2, A and B, the after-action measured to half-relaxation are 0.32 and 0.29 sec. respectively, while in C it is only 0.27 sec. This provides direct evidence that the smaller degree of shortening occurring when only one root is stimulated as compared with that when both roots are stimulated has increased the extent of the response in the fibres participating. Another consideration, namely, that the gastrocnemius is not a parallel-fibred muscle, may also be

recalled.\* When only a portion of the fibres are responding the resultant of force would be greater for the fibres participating because, owing to the smaller shortening, they would be pulling less diagonally. Dr. Katz (1925), working under Prof. A. V. Hill, has found that very little discrepancy exists between the heat production of gastrocnemius when the roots are first stimulated singly and then together, which also supports the conclusion arrived at in the present experiments.†

A possible source of error mentioned by Sherrington (1892, p. 710) in experiments involving stimulation of roots, is escape of current, a point recently investigated in detail by Kato (1924). The freed portion of the lumbar roots in a 50-gm. frog is seldom more than 20 mm. in length, and if the nerve is moist a stimulus which is slightly over-maximal will "wander" to the junction of the two roots. When electrical responses are recorded simultaneously with the mechanical this source of error can be carefully controlled. One record was obtained in which the second and fourth stimulus to root VIII escaped to root IX, and in this way produced as large an action current as those which occurred at corresponding points in the ascent when both roots were stimulated simultaneously. This escape was reflected in the irregularity and increased height of the mechanical record. The stimuli were 1 cm. coil distance above maximal, and there 8 mm of moist nerve between the most distal electrode and the junction of the two roots. This emphasises the liability of error in such experiments when the possibility of current escape is not carefully controlled.

With these considerations in mind, the fatigue experiments recorded by Cattell and Stiles and Beritoff (1924, *b*) seem open to the alternative explanation mentioned above. They found in keeping with Panizza and Kronenberg that when one root is stimulated to exhaustion the muscle will still respond vigorously when stimulated through the other. This I would urge, is not necessarily evidence for fatigue having occurred at the motor junction, but merely that the muscle fibres innervated by one root are exhausted and the unstimulated fibres innervated by the other are not.

\* Beritoff (1924, *a* and *b*), however, has examined from this point of view a large number of muscles including sartorius, semitendinosus and gracilis, to which this specific objection, of course, cannot hold. He infers from these and other experiments (Beritoff, 1924, *c* and *d*) that fatigue is strictly localized to the region of termination of the nerve. This is difficult to reconcile with the conductivity of the wave of excitation in muscle.

† An investigation by de Boer (1925) (published while the present paper was passing through the press) on root stimulation of intact veratrinized muscle has led to the same conclusions.

If now one turns to the more general problem presented by Waller's and Lucas' experiments, careful consideration leads to the conclusion that here too fatigue occurs *within the muscle fibre* rather than in the end-plate. This may appear a pedantic distinction, but it would seem necessary for the following reasons: (1) The ordinary fatigue curves of the text-books show progressive modifications of the response of the muscle as a whole, (2) Progressive modifications of the "angle" described elsewhere (1925, c) with increasing degrees of muscular fatigue are also in keeping with these observations, (3) Moreover, if one compares the direct response of a muscle previously completely fatigued for nerve stimulation, its period of relaxation may be 10 or 15 times longer than the fresh muscle (fig. 5). This again indicates fatigue of the individual fibres: (4) Finally, the alternative view is really that urged by Lapicque (1919), for he finds that whereas normally a muscle fibre appears to have the same chronaxie as its nerve (*i.e.*, is "isochronous"), after stimulation through the nerve becomes ineffectual owing to fatigue, the chronaxie of the muscle substance has doubled or trebled its previous value without any alteration in the rheobase (*i.e.*, it has become in his terminology, "heterochronous"). Thus it would appear that while after severe fatigue the *block occurs at the junction*, the reason for its occurrence there is that time relations are altered owing to the accumulation of acid metabolites within the individual muscle fibres themselves. It is difficult in view of this to accept Lucas' (1911, 1917) interpretation of conduction with a decrement in junctional regions, for when a first stimulus is ineffective, the second one may prove adequate owing to its merely prolonging the excitatory process at the end-plate.

#### *Concurrent Stimulation of the Two Roots.*

A number of experiments have been performed in which one lumbar root in a decerebrate frog has been stimulated for a brief interval *during* the response of the other root. In the case of tetanic responses great difficulty has been encountered in obtaining synchronous repetitive excitation of two roots independently. The mechanical response, however, whether stimulation is synchronous or not is invariably clear-cut. When root B is stimulated during the "plateau" of root A, the myograph rises by an amount corresponding to the difference of response between AB simultaneously and A alone (fig. 3). But the electrical responses are naturally difficult to interpret unless completely in phase. Fig. 3 is a record of an unsuccessful experiment, the details of which are described in the legend. The increased size of the electrical responses

despite their altered frequency during the concurrent stimulation is significant as indicating a large proportion of added muscle elements. Also the rise of the myograph cannot be accounted for by mere increase in the rate of stimulation above 50 per sec. (Liddell and Sherrington, 1925, *b*, p. 501 ; Fulton, 1925, *c*, p. 436) for this does not materially increase the tension developed in a motor-nerve plateau. Another feature shown in fig. 3A is the augmentation in the electrical responses during the period following the concurrent stimulation, when the muscle is lengthening. It has been shown elsewhere (1925, *a*) that when a muscle under appreciable initial tension shortens, the successive electrical responses decrease *pari passu* with the muscle length, and from this record it is clear that the reverse is also true namely, *that the size of the successive electrical responses increases progressively as a muscle is lengthened.*

Another feature of these concurrent excitations brought out in fig. 4, in which a twitch of root B has been given during the plateau of root A, is that a tetanised muscle can *maintain greater tension than it can attain.* Thus, after the twitch from root B in fig. 4, the myograph falls, but *not* to the previous plateau level. Having been "assisted" by the fibres in B root, the fibres stimulated by root A can remain more shortened and at a higher tension than they had previously achieved. Conformably with this the successive electrical responses from A root are *smaller* after the stimulation of B than before. In other words the size of the electrical responses in this case *varies with the length of the muscle and not with the tension developed.*

Other variations of the experiments involving concurrent stimulation are described in the legends to the figures. The results are all quite intelligible on the grounds of independent innervation, and with the exception of the 5 to 10 per cent. overlapping in tension developed by the separate roots, all evidence from these experiments points definitely to the absence of plurisegmental innervation of single fibres.

#### *Diffusability of "Fatigue" Products.*

The question raised by Sherrington (1892) as to whether fatiguing one lumbar root affects the response of the other root has been examined in a small number of experiments (four) by the following method. The "angle" of the twitch provides, as we have seen, a very delicate index of the "condition" of the muscle, especially with regard to fatigue. A twitch has therefore been recorded by stimulation of the "test" root ; immediately after this, and on the same photographic plate, the other root is fatigued to exhaustion, and

after this, another single response is immediately recorded from the "test" root. The result of such an experiment is shown in fig. 6. In the lower record (C) root IX was stimulated by a single break shock, and immediately after this root VIII is tetanised. The first part of the tetanic response is shown. A later "sample" of the response after an interval of 55 sec. is shown in the middle record. This record shows gradual diminution of the mechanical response. After another two-minute interval, the fibres innervated by the VIIIth appeared nearly exhausted, and in the last record of fig. 6 the end of the response is shown, followed immediately by another twitch from IX. It will be seen that there is a small relaxation after cessation of the stimuli showing the extent of the exhaustion. The response of root IX in this record has an "angle" even sharper than that of the first response, but the relaxation is slightly less rapid. In this preparation after the fatigue, a distinct hyperpnœa followed which lasted five or six minutes. "A condition which does more modify the result is the maintenance or removal of the circulation. When instituted on an excised muscle the degree to which fatigue produced from the 'fatiguing' root influences the response obtainable from the test root is more marked. the influence becomes apparent earlier and persists longer." (Sherrington, 1892, p. 711.) Thus, in one preparation in which the circulation had stopped, the second "angle" of the test root after the other root had been exhausted was nearly obliterated and relaxation was considerably less rapid. However, the modification in the "angle" and the relaxation was far less in extent than that shown, fig. 5, B, in which the whole muscle had been stimulated to exhaustion and then caused to respond directly. This would appear to be direct evidence that the majority of fibres stimulated through the test root are "fresh" fibres, and not merely fatigued fibres stimulated through an accessory end-plate—a conclusion which harmonizes with the recent findings of Samojloff (1924) and de Boer (1925).

It is clear from the evidence provided by fig. 6 and other similar records that if the circulation is active, acid metabolites, when extensively accumulated in one fibre, do not readily diffuse into adjacent fibres. In view of the cutting of the vasomotor nerves in such experiments, with resulting vasodilation and tendency to œdema, it is likely that even the slightly delayed relaxation observed in the second twitch in fig. 6 would not have occurred in an intact animal. Acid metabolites of muscle appear therefore to be readily diffusable through the capillary walls into the blood stream, but not through the sarcolemmal sheaths into surrounding fibres. This may be regarded as of importance in the co-ordination of reflex activity. Forbes and Cattell (1924), Fulton and Liddell



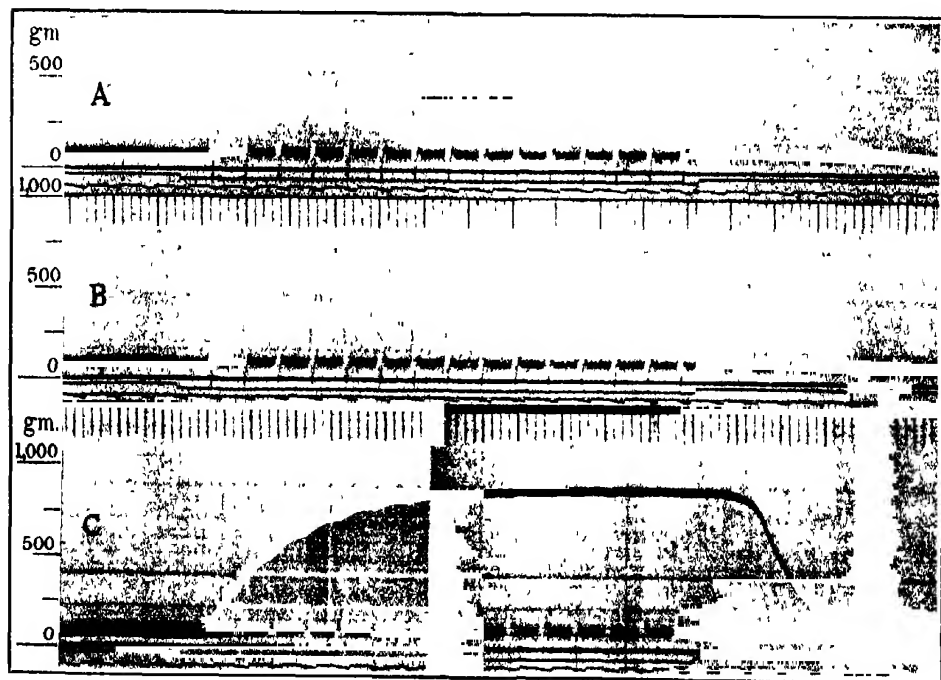
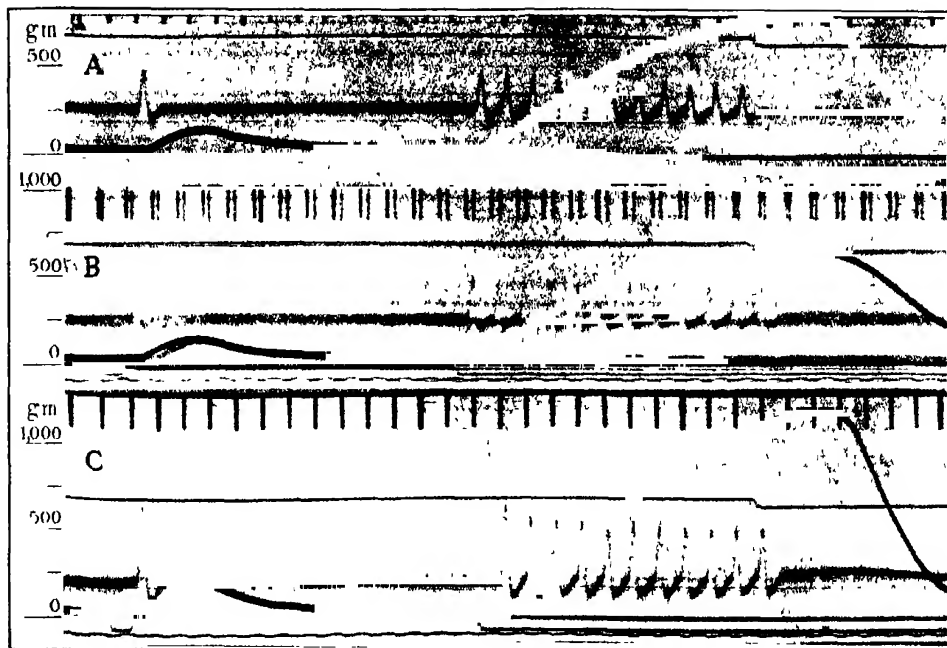
(1925, *a* and *b*) and others, have directed attention to the fact that in certain forms of reflex activity the all-or-nothing contraction of the individual muscle fibres is asynchronous. In the case of the stretch-reflex, which Liddell and Sherrington (1924 ; 1925, *a*) regard as a form of postural tonus, the responses are also asynchronous and are maintained for long intervals (Sherrington and Liddell, 1924, p. 224) without fatigue—far longer than a maximal motor tetanus could be maintained. It is evident from this that when the individual fibres of a muscle respond asynchronously, one after another, and possibly in rotation, as Forbes (1922, p. 404, *cf.* Fulton and Liddell, 1925, *b*) has suggested, it is of great advantage to the muscle as a whole for the acid metabolites produced in active fibres *not* to diffuse into resting fibres. This helps to account for the relatively high resistance to fatigue possessed by the muscles engaged in postural activity.\*

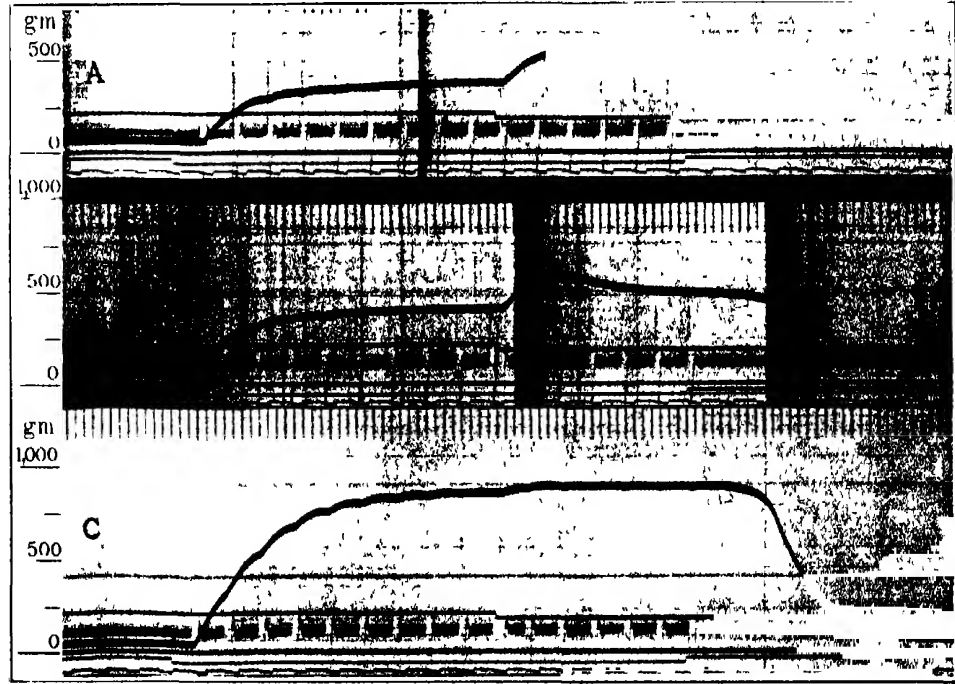
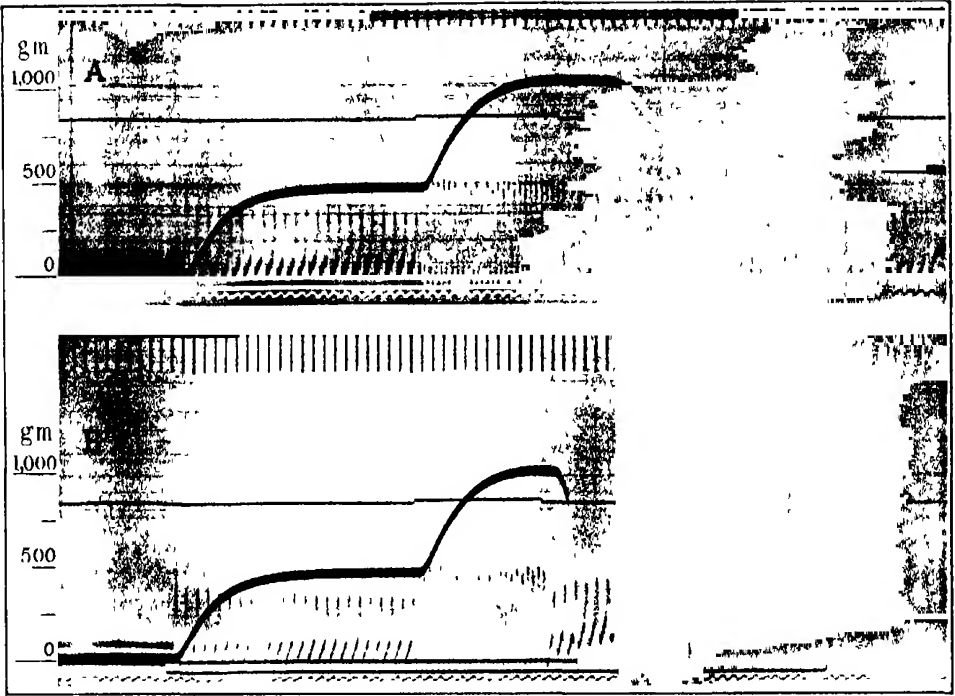
*Résumé.*—Plurisegmental innervation of individual muscle fibres in the frog is unlikely for the following reasons : (1) over-lapping of the sum of the responses from roots VIII and IX stimulated singly above that when the two are stimulated simultaneously is, when precautions are taken against current escape, seldom higher than 10 per cent. either for the action current or for mechanical tension. (2) This degree of overlapping may be accounted for without assuming double innervation of the fibres, by the fact that more contractile energy is liberated in the individual fibres of a muscle the less it shortens. (3) Finally, plurisegmental innervation is unlikely on embryological grounds, and on the grounds of probability. The liability of current escape in these experiments is emphasised.

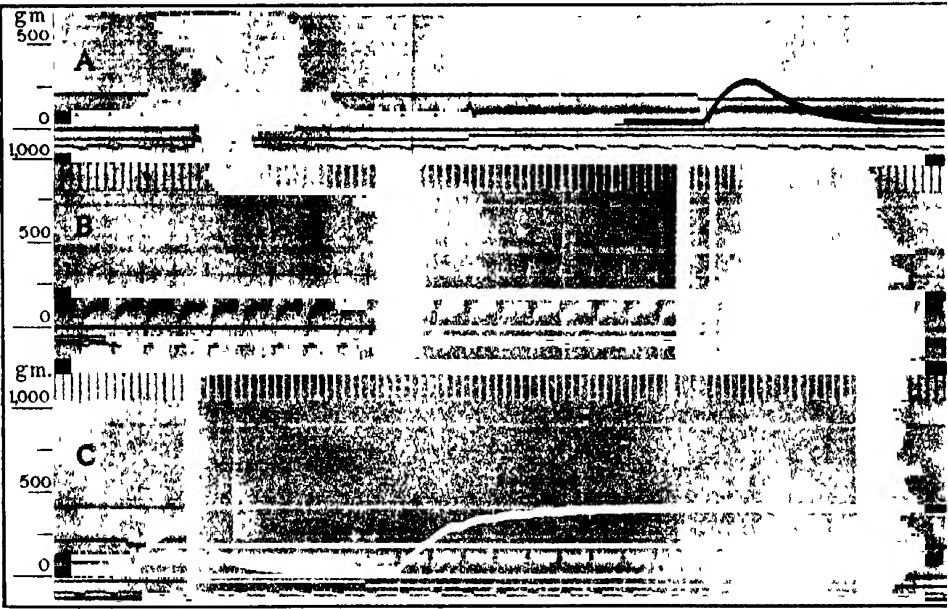
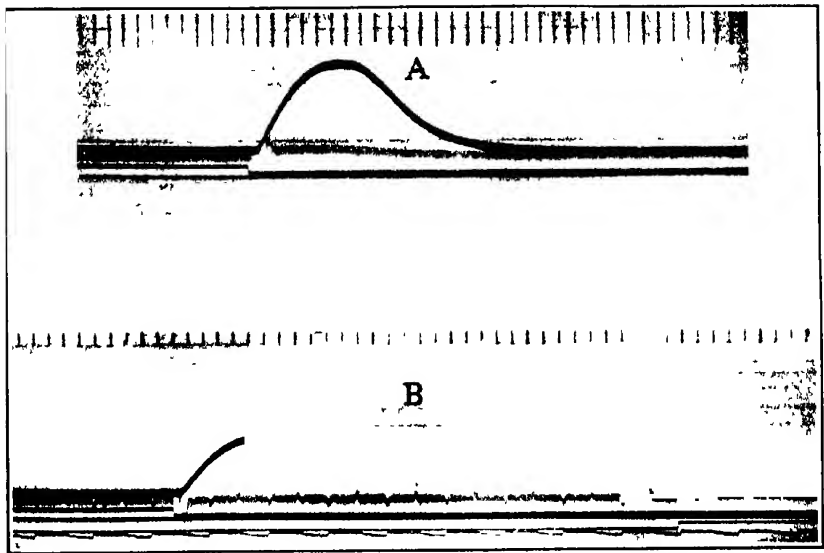
The view that fatigue occurs not at the end-plate, but as a result of accumulation of acid metabolites within the individual fibres is urged.

Provided the circulation is active, fatiguing to exhaustion of one root has little influence upon the response of the other root as judged by the "angle." From this it may be inferred that in the normal animal acid metabolites produced in active fibres do not diffuse into resting fibres.

\* A discussion of the possibility of sympathetic influence in the activity frog muscle will be found in Cobb's (1925) paper.









DESCRIPTION OF PLATES 32, 33 AND 34.

PLATE 32.

FIG. 1.—Responses of the intact gastrocnemius stimulated through its lumbar roots. Temperature 16°; time above in 0.02 sec. From above downwards are “Off” key; string of galvanometer; myograph; line of zero tension; “On” key, vibrator showing rate of stimulation.

A. Root VIII stimulated alone; B. Root IX stimulated alone (doubly exposed), C. Roots VIII and IX stimulated simultaneously. Two-minute intervals were allowed between each response. 2 cm. vertical distance equal to 600 gm. tension. Weight of frog 35 gm. Rate of stimulation 50 per sec. The strength of stimulation was in each response 0.5 cm. coil distance above maximal, which, provided monophasic shocks (break) are used, does not appear to spread for more than 1 to 2 mm. down the nerve.

FIG. 2.—A repetition of the responses shown in fig. 1 from another preparation (25 gm. frog) at 13° stimulated at 14 per second. Conditions otherwise as in fig. 2. In this record there is 4 to 5 per cent. overlapping electrically between A and B taken singly and AB taken simultaneously (in C). Note the overlapping in size of the first mechanical responses of the ascent of the tetani in A and B as compared with C (cf. Table I). 600 gm. — 2 cm. vertical distance.

PLATE 33.

FIG. 3.—A record of an unsuccessful experiment in which the VIIIth root was stimulated continuously and the IXth for only a short interval indicated by the upper signal. During the interval in which both stimuli were being delivered at once the mercury key used to separate make from break shocks failed to separate owing to the primary of the two coils being connected in series. The synchronization of two sets of independent stimuli presents a difficulty which has not yet been overcome. Inasmuch, however, as doubling the rate of stimulation during the plateau of a completely fused tetanus does not materially increase the tension already attained, the increase of tension in this response is clearly to be attributed to the recruitment of added muscle units. This is also borne out by the increased amplitude of the electrical responses despite the fact that their rate is doubled. In the lower response the tendon broke at the height of tetanus.

Note in A the increasing size of the electrical responses during the relaxation (*i.e.*, lengthening) from the second stimulation. This shows that the electrical responses increase when the fibre is caused to lengthen. In B after the tendon has broken, note the small size of the electrical responses. Here the muscle is presumably at its shortest length.

FIG. 4.—Responses of intact gastrocnemius showing the effect stimulating one lumbar root by a single shock when the other root is being tetanized at 14 per sec. (monophasic stimuli). Time above = 0.02 sec., temperature = 12.5°. A. Root VIII tetanized as indicated by lower signal; during the plateau root IX is stimulated by a single breakshock shown by the upper signal. The action current produced by this break shock fell during the down stroke of one of the action currents of the tetanus, but it shows itself by a brief “hesitation” in the string 1.3 mm. after closure of the break key. Note the diminished size of the last five action currents and also the fact that

the myograph does not return to the previous plateau tension ; B. Root VIII tetanized as in A, but in this record the single shock has been applied in the region of the thigh to the whole sciatic trunk. Note the increased size of the response so produced and also the difference in conduction time as indicated by the latency of the electrical effect in A and B. The diminution in size of the electrical responses after the "twitch" is slightly more marked in this record than in the preceding ; C. Both roots tetanized simultaneously followed by a single shock applied as in B in the thigh. Note the small effect produced. This indicates that all or nearly all the fibres in the lumbar roots were being stimulated and is therefore a control to the stimuli in A and B being maximal

Same preparation as in fig. 2.

#### PLATE 34.

FIG. 5.—The response of the same muscle before (A) and after (B) severe fatigue. In the upper response the muscle was fresh. It was then tetanized until it ceased to respond to nerve stimulation, and then stimulated directly. Such a direct response is shown in the lower record (B). The escape of the stimulating current is in the same direction to the action current. The periodic movement of the stung in B is due to escape from the tetanizing stimuli still being applied to the nerve.

FIG. 6—Responses of the intact gastrocnemius stimulated through its lumbar roots showing that one root may be stimulated to exhaustion without greatly affecting the response from the other root. A. Root IX was stimulated by a single break shock shown by the signal. This is followed by the beginning of a tetanizing stimulus at 28 per sec. applied to the VIIIth root. B. A "sample" of the responses during the exhaustion of the VIIIth root taken 55 sec. after the end of the last record. C. A continuation 2 minutes later of the same tetanus after the mechanical response had fallen to a negligible value. This tetanus is ended about half-way down the plate, after which there is very slight relaxation and then another break shock is delivered to the IXth root. It will be seen that the "angle" in this second twitch is if anything more precise than that in A. The relaxation, however, is slightly less rapid. (Cf. fig. 5b.)

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*The Surface-Tension Theory of Muscular Contraction.*

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It was shown by Bernstein (1) in 1908 that the maximum mechanical response in a muscle twitch is greater at a lower temperature. Since surface tension decreases as the temperature is raised, this observation was regarded as strong evidence in favour of the theory that "changes in surface tension are a controlling factor in the development of the energy of muscular contraction" (Bayliss (2), p. 448); other physical effects such as osmotic pressure and "Quellung" were, according to Bernstein, excluded since these increase as the temperature is raised. If it had been shown at the same time that the total energy liberated in a muscular contraction was independent of temperature, the mechanical energy alone varying, this might indeed have been regarded as in favour of a surface-tension theory. Actually, however, the total heat set free in a twitch decreases as the temperature is raised, in just the same way as does the tension; indeed, there is a very constant relation between the two, so that *for a given liberation of total energy, i.e., for a given chemical change, the tension energy set free is independent of the temperature.* Bernstein's observation, therefore, gives us no grounds for concluding that the development of the mechanical response in muscle is due in any way to changes of surface tension. To put the matter in terms of lactic acid, a given production of lactic acid is accompanied by the same rise of tension whatever the temperature.

If further evidence be required against the deduction from Bernstein's observations it is supplied by the fact that in a tetanic contraction the tension developed and the heat set free are both greater, and not less, at the higher temperature. When a frog's muscle is maintained in a constant state of contraction by a succession of stimuli, the tension is not lower at a higher temperature, as it should be on the surface-tension theory, but appreciably higher. Another explanation of these facts has been given by Hartree and Hill (3, p. 141).

Such considerations do not in themselves dispose of the hypothesis that muscular contraction is due to a change of surface tension; they merely

invalidate the argument brought forward in its favour. It was still possible that a change of surface tension might, in some way, be fundamentally the cause of muscular activity. That this is not so is shown by the following considerations.

A change of surface tension, properly so-called, can be produced by one cause only, namely, by the spreading out of a layer of some chemical substance upon the surface concerned. The thinnest film capable of causing a definite change is one involving a single layer of molecules covering an appreciable fraction of the area. It is impossible to imagine that a collection of individual molecules scattered independently upon a surface with large spaces between them, could produce any considerable change in the energy of that surface. Only those parts of the surface which were covered or approximately covered with a layer of some other substance could be expected to show any alteration from their usual condition. The layer required to produce the change in surface energy might be *more* than one molecule thick, but it could not well be less. Let us assume, therefore, that muscular contraction is due to the formation of a layer of some chemical substance, at least one molecule thick, spread out upon certain active surfaces in the fibres.

In the anaerobic phase of muscular contraction, during which the tension rises and falls again, there is only one chemical substance known for certain to be produced, namely, lactic acid. The  $\text{CO}_2$  set free in this phase is derived simply from pre-formed bicarbonate reacting with the acid. Oxidative processes do not come into question, since the muscle can function equally well for a while, even in the complete absence of oxygen. Possibly changes may occur in the phosphate present in the muscle (Embden (4) ); if, for example, lactic acid be derived from a hexose di-phosphate, the liberation of each molecule of lactic acid would be accompanied by the simultaneous liberation of a molecule of phosphate. There is no evidence, however, that any chemical body is produced during anaerobic muscular activity in greater amount than the lactic acid known to be set free. If some substance be required to produce a change of surface tension large enough to account for the mechanical response, it is natural to look to lactic acid.

Mines (5), indeed, in 1913, brought forward reasons for regarding the production of lactic acid as responsible for the changes of surface energy which underlie muscular activity. His evidence, however, was merely of a suggestive and not of a quantitative type, and it is desirable to consider quantitatively the implications of the surface-tension theory. Fortunately, the data are available by which a rigorous quantitative test can be applied.

During a muscle twitch there is a constant relation between the force developed and the amount of lactic acid set free. For a given force developed the area can be calculated which the corresponding lactic acid would occupy if spread out in a film one molecule thick. The surface tension existing in this area can be evaluated, on the assumption that the force developed by the muscle is due to surface tension. The value so calculated proves to be impossibly high; therefore surface tension cannot be the cause of the mechanical response. This is the argument developed below, and the data on which it is based can be derived either (a) indirectly, from observations on the relation between heat-production and tension in a single twitch, or (b) directly, from Meyerhot's experiments on the relation between total tension and total lactic-acid production in a long series of responses.

(a) *Single Twitch*.—In a muscle twitch a constant relation exists between the heat produced and the tension developed. When a frog's muscle is stimulated by an induction shock, or by a tetanus of short duration, at a length not far different from its resting unloaded length, and provided that it be in reasonably fresh condition, the relation is  $H/Tl = 0.2$  approximately. Here  $H$  is the heat production reckoned in ergs,  $T$  is the force developed in dynes, and  $l$  is the length of the muscle in centimetres. The physical basis of this relation is as follows. If we consider only a single muscle fibre, it is obvious that when it gives a maximal response the heat production—other things being equal—must be proportional to its length. If, moreover, there be  $n$  times as many fibres, clearly the heat production will be  $n$  times as great, as also will the tension. Thus, provided that the tension and heat production are altered merely by changing the number of fibres responding to a stimulus, and by varying their length, it is obvious that  $H/Tl$  should be constant. This quantity, however, is constant not only under these conditions of varying the number of fibres and their length, but also when the response of the individual fibre is altered. A change of temperature which has a considerable effect upon both  $H$  and  $T$ , leaves the ratio  $H/T$  unchanged. The duration of the stimulus, up to a certain limit, which is greater in the case of a tortoise's muscle than of a frog's muscle, increases both  $H$  and  $T$  but leaves their ratio practically unaltered. Even a change of initial length of the stimulated muscle, over a range of about 10 per cent. either way, has little effect upon  $H/T$ , since this quantity has a minimum value in the neighbourhood of the resting unloaded length.

In a twitch, or in a very short tetanus, of a frog's muscle, Hartree and Hill (6) found at all temperatures the same value of  $H/Tl$ , namely 0.21. The curves

relating  $H/Tl$  to the duration of a tetanus, at different temperatures, all came down to the same point on the axis, corresponding to a value of  $H/Tl = 0.21$  for zero duration of stimulus. The muscles of the frog are very rapidly moving ones, and it is striking that in recent experiments the very slowly moving muscles of a tortoise\* give almost precisely the same value, as the following numbers show : —

Experiment 1 . . . . .	13.6° C.	0.19
	24.4° C.	0.18
Experiment 2 . . . . .	14.8° C.	0.23
	24.5° C.	0.19
Experiment 3 . . . . .	14.3° C.	0.21
Experiment 4 . . . . .	13.5° C.	0.22
Experiment 5 . . . . .	24.2° C.	0.21
Experiment 6 . . . . .	15.5° C.	0.21
	25° C.	0.23
Experiment 7 . . . . .	18° C.	0.20
Experiment 8 . . . . .	14.5° C.	0.17
	20.1° C.	0.18

Mean value, 0.202.

It would seem, therefore, that in this ratio  $H/Tl$ , for short durations of stimulus, we have something approximating to a natural constant for the case of striated muscle.

Let us imagine that we have a muscle fibre of length 1 cm. exerting, in a maximal twitch carried out isometrically at its resting unloaded length, a force of 1 dyne. Then the heat evolved will be 0.2 erg. From this heat we may calculate as follows the lactic acid set free. According to a variety of observers [A. V. Hill (7), Peters (8), and Meyerhof (9)] there is a liberation of about 370 calories when 1 gm. of lactic acid is produced in muscle. Of this only about 296 calories fall in the initial phase; the rest is delayed (13). One calorie is  $4.18 \times 10^7$  ergs, so that the liberation of 1 gm. of lactic acid is accompanied in the initial phase by a heat production of about  $1.24 \times 10^{10}$  ergs. From this, assuming that 0.2 erg of heat is liberated when a tension of 1 dyne is developed in 1 cm. length of muscle, we may calculate that the latter

\* *Added in proof*—These experiments on tortoises were made in winter and spring. Recent experiments made by Mr. W. Hartree at Cambridge during the summer have given a considerably smaller value, as low as 0.15 or even 0.10. Such a seasonal variation would seem to be of great interest, and will be further investigated. Its existence is a complicating factor, but does not invalidate the general argument which follows.

circumstance is accompanied also by the formation of lactic acid to the extent of  $1.61 \times 10^{-11}$  gm.

(b) *Long Series of Twitches*.—Approximately the same result can be obtained from certain independent facts due to Meyerhof (10). Meyerhof carried out experiments on frog's muscles, in which he compared the total tension developed in a long series of muscle twitches with the total lactic acid set free. He expressed his results in terms of what he called the isometric coefficient for lactic acid. His isometric coefficient (K) he defined as follows :

$$K = \frac{(\text{total force developed in kgm. weight}) \times (\text{length in cm})}{(\text{mgm. of lactic acid produced})}$$

Expressing everything in absolute c.g.s. units Meyerhof's results may be stated in the form : (gm. of lactic acid produced) =  $(Tl/K) \times 1.02 \times 10^{-9}$ . For fresh semi-membranosus Meyerhof found a value for K of 78; for unfatigued gastrocnemius one of 123. The difference between the two types is due to the fact that in the gastrocnemius the fibres do not run the whole length of the muscle. Certain independent data\* show us that on the average the fibres in gastrocnemius run only 63 per cent. of the length of the whole muscle. It is necessary, therefore, to multiply the isometric coefficient for gastrocnemius by 0.63 before comparing it with that for adductors. This gives 77.5, practically the same as the value for the other muscles. Applying the above formula we then find (gm. of lactic acid) =  $Tl \times 1.31 \times 10^{-11}$ . Thus, accepting Meyerhof's data, we find that a muscle fibre developing a force of 1 dyne in 1 cm. of its length, must liberate  $1.31 \times 10^{-11}$  gm. of lactic acid. The agreement of this value with that ( $1.61 \times 10^{-11}$  gm.) calculated above from independent data is striking testimony to the general validity of our argument. We will assume a mean value therefore, namely,  $1.46 \times 10^{-11}$  gm. of lactic acid to be liberated when a fibre 1 cm. long develops a force of 1 dyne.

Recently, in a private communication, Dr. A. C. Redfield, of Harvard University, has informed me of certain experiments of his own, somewhat analogous to those of Meyerhof (10) from which a value of the same quantity can be deduced for the case of the *heart muscle of the tortoise*. The experiments were not intended primarily for this calculation, so the results must be taken only as approximate. The value is  $2.3 \times 10^{-11}$  gm. of lactic acid per dyne per cm. This, although larger than those deduced above, is of the same order

\* The ratio  $\frac{T \times l}{(\text{area of tension-length curve})}$  has for gastrocnemius an average value (according to Meyerhof) of 13.5, and for sartorius, semi-membranosus and gracilis, 8.5. This, as shown by Mashimo (11) is due to the fibres of gastrocnemius not running the full length of the muscle. Thus, the ratio (fibre length) : (muscle length) is 8.5 : 13.5, i.e., 0.63.

of quantities, and supports the belief that the lactic acid mechanism is the same in cardiac as in skeletal muscle.

Since this paper was completed Prof. Meyerhof has called my attention to the experiments of Matsuoka (15, p. 585), who found in *sartorius* an appreciably higher value of K, averaging 108 for muscles in Ringer's Solution, or in nitrogen gas after KCN. This makes the divergence between the two methods of calculation rather wider, but does not affect the general tenour of the argument.

An atom of hydrogen weighs  $1.66 \times 10^{-24}$  gm. A molecule, therefore, of lactic acid is 90 times that quantity, namely,  $1.49 \times 10^{-22}$  gm. Thus in the process of developing a tension of 1 dyne in 1 cm. length of muscle, the number of molecules of lactic acid liberated is  $\frac{1.46 \times 10^{-11}}{1.49 \times 10^{-22}}$ , that is to say, very nearly  $10^{11}$  molecules.

Let us calculate the area occupied by this number of lactic acid molecules if spread out in a mono-molecular film. We may proceed in two ways, (a) and (b), both, however, leading approximately to the same result.

(a) The density of lactic acid in mass at  $15^{\circ}$  C. is 1.25. Let us attribute the same density to the lactic acid molecule. Its volume then will be  $\frac{1.49}{1.25} \times 10^{-22} = 1.19 \times 10^{-22}$  c.c. Let us imagine for simplicity of calculation that the molecule is cubical in shape: then the area of one of its faces is the  $2/3$  power of the last named quantity, namely,  $24.2 \times 10^{-16}$  sq. cm.

(b) According to N. K. Adam (12) the area occupied by a fatty acid molecule forming part of a condensed film on the surface of water is about  $21 \times 10^{-16}$  sq. cm. The area occupied by a lactic acid molecule cannot be far different from that of the cross-section of the hydrocarbon chain of a fatty acid. Adam's value, therefore, agrees closely with that calculated from the density.

We will assume, then, that a single lactic acid molecule occupies an area of about  $21 \times 10^{-16}$  sq. cm. Hence  $10^{11}$  of them would occupy an area of  $2.1 \times 10^{-4}$  sq. cms. Thus a muscle fibre of length 1 cm., developing a force of 1 dyne in a twitch, would liberate sufficient lactic acid molecules to cover completely an area of  $2.1 \times 10^{-4}$  sq. cm.

Now the tension developed by a muscle exists throughout its length. The area, therefore, over which the lactic acid has to spread must be 1 cm. long, otherwise the tension would not be developed throughout the whole length of the fibre, as by hypothesis it is. Hence the area occupied by the lactic acid molecules, being 1 cm. long, must be  $2.1 \times 10^{-4}$  cm. wide. We may regard this area, if we like, as that of a small cylinder running from one end to the other

of the fibre ; the circumference of this cylinder then is  $2.1 \times 10^{-4}$  cm., that is to say,  $2.1 \mu$ .

Now by hypothesis our muscle has developed a force of 1 dyne. If that force be due to surface tension exerted along the surface of a cylinder of circumference  $2.1 \times 10^{-4}$  cm., the coefficient of surface tension  $\gamma$  must be such that  $2.1 \times 10^{-4} \gamma = 1$ . In other words, the coefficient of surface tension required is 4,800 dynes per cm. Hence, on the hypothesis that our lactic acid has formed a mono-molecular layer, the rise of surface tension produced by it must be 4,800 dynes per cm. This is about 66 times the surface tension of water, 200 times the surface tension of alcohol, eight times the surface tension even of mercury ; the surface tension of a water-olive-oil interface is only about 21 dynes ; clearly the value of 4,800 dynes is physically impossible.

It is unreasonable, therefore, to regard a mono-molecular layer of lactic acid as capable of causing a change in surface tension large enough to explain the actual force developed by a muscle in a twitch. With a layer thicker than a mono-molecular one, the difficulty is only enhanced. It is hard in the muscle fibre to imagine a *change* of surface tension larger than that existing already between water and olive oil. With such a coefficient of surface tension we must, if we wish to obtain the requisite surface energy, employ a surface which is 230 times as large as that which would be occupied by a condensed mono-molecular film of the lactic acid actually liberated. It is impossible to suppose that a change in surface tension as large as that existing between water and olive oil could be caused by molecules of lactic acid, dotted at wide intervals about a surface, occupying only 0.4 per cent. of its area. Clearly, the surface-tension hypothesis in its simple form is untenable.

It may be objected that we are not justified in assuming that lactic acid is the body which produces the change of surface tension required. Something, however, must produce the effect, presumably in a layer not less than one molecule thick, and the above calculation shows that if this "something" is to cause the response of muscle it must be liberated in amount about 230 times as great as is lactic acid. Otherwise, it could not occupy the requisite area with any reasonable coefficient of surface tension. There is not, however, the slightest evidence that any substance is liberated in a muscle twitch in amounts hundreds of times as great as that of lactic acid. We seem, therefore, to be reduced to the conclusion that a change in surface tension, properly so-called, is not the cause of muscular contraction.

Many facts, however, lead us to the view that the reactions which underlie the response of muscle occur at surfaces, or interfaces, in the fibre. There are

other forces besides that of surface tension which might act at such interfaces. In a subsequent paper, W. E. Garner discusses the possibility of an action of lactic acid upon the surface layers of a system of liquid crystals localised inside the muscle fibres. The presence of such a system could explain many of the well-known properties of muscle. Or, again, we may imagine that the fibrils visible inside the fibre consist of protein substances negatively charged with a cloud of positive ions around them. Each cylinder of protein, negatively charged with its attendant cloud of positive ions, would in effect constitute an electrical condenser. Such a condenser would be in a state of strain under the mutual repulsion of the elements of charge making up its plates. The sudden liberation of lactic acid in the neighbourhood of the negatively charged protein surface would cause a discharge of the condenser by the formation of sodium lactate and un-ionised protein. The mutual repulsion of the charges would be abolished and the condenser would tend to shorten. The force developed in such a condenser suddenly discharged can be calculated provided that we know its dimensions. With certain possible assumptions the force developed, so calculated, is large enough to be reconciled with the data at our disposal. The hypothesis, however, is at present too vague to deserve further consideration.

One further comparison may be of interest, viz, that of the surface available in muscle at the interfaces visible under a microscope, with the area of a mono-molecular film of the lactic acid set free. A homogeneous frog's muscle, such as the sartorius, is composed of fibres about  $50\mu$  in diameter. A frog's sartorius weighing 200 mgms. and 3 cm. long can, under favourable circumstances, develop a force of about 150 gm. weight, about 150,000 dynes. Assuming the muscle to have the same specific gravity as water, calculation shows that its area of cross-section is 6.7 sq. mm. Per square millimetre, therefore, it can develop a maximum force of about 22,000 dynes. In this square millimetre there must be about 400 fibres each of  $50\mu$  diameter. Each fibre, therefore, must be able to develop a force of about 55 dynes. Its circumference is  $157\mu = 0.0157$  cm. Hence per centimetre of circumference the maximum force developed by the fibre is  $\frac{55}{0.0157} = 3,500$  dynes. It is striking that this value is of the same order of quantities as that calculated per centimetre of edge on the hypothesis of a mono-molecular film of lactic acid.

This result can be expressed in another way. In the maximal contraction of a frog's muscle the amount of lactic acid set free, if spread out in a film 1 molecule thick, would be able to occupy an area about equal to the surface



of all the muscle fibres of which the muscle is composed. Whether this result is a coincidence or not it is impossible at present to say; it suggests that the surface of the muscle fibre itself may be the place at which the lactic acid works.

Inside the muscle fibres, visible under the microscope, are the muscle fibrils, stated to be about  $1\mu$  in diameter, according to Bernstein (14) about 31 million of them to the square centimetre of muscle. In 1 c.c. of muscle, therefore, it may be calculated that the area of all the fibrils which it contains is about 20,000 sq. cm. In our calculation above we have assumed that the muscle can exert a force of 22,000 dynes per square millimetre. This is 2.2 million dynes per square centimetre. Thus, for every centimetre of edge of fibril the maximum force exerted by the muscle is about 110 dynes,\* only about one-thirtieth of that calculated for the fibres. Expressed in another way, in the maximal contraction of a muscle the amount of lactic acid set free would be sufficient to cover, in a mono-molecular film, only one forty-fourth of the area of the ultimate fibrils visible inside the fibre. If, therefore, some hypothesis similar to that of Garner's be correct, we may suppose that in a maximal contraction about one forty-fourth of the area of the muscle fibrils is occupied by lactic-acid molecules suddenly set free, with the consequent effect upon the lattice of liquid crystals there situated.

### *Summary.*

1. It is possible, from two independent sets of data, to calculate the amount of lactic acid liberated when a muscle fibre 1 cm. long develops a force of 1 dyne. The mean value found is  $1.46 \times 10^{-11}$  gm. This is very nearly  $10^{11}$  molecules.

2. The area occupied by this number of lactic-acid molecules, if spread out in a continuous mono-molecular film, is about  $2.1 \times 10^{-4}$  sq. cm.

3. If the mechanical response of muscle were due to a change of surface tension caused by a film of lactic acid, the coefficient of surface tension required would be about 4,800 dynes per centimetre. This is about 230 times the tension of a water-olive-oil interface, clearly an impossible value.

4. Other reasons are given for regarding the surface-tension hypothesis of muscular contraction in its simple form as untenable.

5. The lactic acid liberated in a maximal contraction of a frog's muscle in good condition, if spread out in a continuous mono-molecular film, would occupy

\* This is still too large for a surface-tension theory, as Bernstein (14) pointed out. Some still finer structure is required on that theory.

an area about equal to that of the surface of the fibres composing the muscle, or to 2 per cent. of the surface of the ultimate fibrils.

6. Reckoned per gram. of muscle the amount of lactic acid liberated in a maximal contraction of a frog's muscle is about 0.033 mgr., which would occupy an area of about 470 sq. cm.

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*The Development of Blood Plasma.—Part I. The Genesis of Coagulable Material in Embryo Chicks.*

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Although much attention has been given to the embryonic development of blood corpuscles, the genesis of the coagulable constituents of the plasma has escaped notice. Knowledge of this subject appears to be limited to the observation of Boll (1) that the blood of chick embryos does not clot before the twelfth to fourteenth day of incubation; and of Emmel, Levison and Fisch (2) that the blood of late pig embryos clots more slowly than that of adult pigs.

We shall describe the successive appearance in chick embryos of those constituents of the blood plasma which are associated respectively with the preservation of the fluidity of the blood and with its capacity to clot. We shall also briefly discuss the significance of the facts recorded.

*The Methods and Terms Employed.*

Blood was obtained from the larger vessels of the yolk sac and allantois by aspiration through a finely-drawn Wright's pipette. Advantage was taken of the fact that in embryos aged 12 days and upwards, the allantoic vessels are adherent to the inner shell membrane. On removing the shell small hæmorrhages occur outside the membrane; blood can thus be obtained without contamination with the contents of the egg. Plasma was obtained both by centrifuging and by the settling out of the corpuscles. Care was taken to avoid contact with damaged tissues, as cell detritus clots the blood of older embryos. Observations were made both at room temperatures and at 40°. Reagents were added by means of a graduated Wright's pipette.

"Commencement" of clotting was noted when the first visible departure from fluidity was observed, other than the formation of minute filaments on the surface of the blood. "Completion" was recorded when the vessel containing the blood could be inverted without spilling. In early embryonic blood the clots formed are often loose in texture and remain in the fluid without adhesion to the containing vessel. In these cases completion of clotting was

noted when no subsequent increase in the size of the clots was apparent. Clotting in crops was also observed. The formation of the last clot of the series was taken as the completion of coagulation. When clotting appeared to be absent to the naked eye microscopic examination was used as a control. In no case were fibrin filaments found when coagulation was invisible to the naked eye.

With the exception of fibrinogen, the plasma constituents which participate in coagulation have not been isolated in a state of purity, but certain tests are generally employed for their recognition. Of these we have used the following, viz. (α) *Fibrinogen* :—Coagulation on heating in glass vessels from 56° to 60°, and by the addition of both thrombin and fresh serum at room and body temperatures. Precipitation by half-saturation with sodium chloride, and by one-fifth saturation with ammonium chloride. (β) *Prothrombin* :—Clotting at room or body temperatures by the addition of tissue detritus and a few drops of calcium chloride. The tissue detritus was prepared by grinding tissue from recently dead embryos in 0·86 per cent. sodium chloride. Control experiments showed that inert particles did not act similarly. (γ) *Thrombin* :—The appearance of clots on recalcifying oxalated embryonic plasma and the clotting of solutions of fibrinogen. (δ) *Ionised calcium* :—The prevention of clotting both at room and body temperatures by the addition of 0·1 per cent. potassium oxalate, and the restoration of coagulability by the addition of sufficient calcium chloride to neutralise the added oxalate. (ε) *The coagulant material of cell and tissue detritus* (The thrombokinas of Morawitz (3), or cytozyme of Fuld and Spiro (4) and Bordet (5)) :—The embryonic tissue detritus and filtered normal saline extracts of ground-up tissue were added to the plasma and its influence on coagulability noted. (ζ) *Globulin, other than fibrinogen* :—Precipitation both by neutralisation and by the addition of an excess of distilled water. When fibrinogen was present it was removed, both by heating to 60° and by coagulating with thrombin. Both solutions were then examined. Too little material was available to permit of testing by dialysis. In view of the conclusion of Sørensen (6) that it is not possible to separate euglobulin and pseudoglobulin by salt-precipitation, attempts were not made to separate the serum globulins into components. (η) *Material protective against the inception of clotting* :—The work of Pickering and his collaborators (7, 8, 9) indicates that there exists in the circulating blood material which prevents the onset of coagulation so long as the surface conditions of the plasma are undisturbed. An excess of such material was assumed to exist in embryonic plasma when the coagulation of human blood

was inhibited by mixing it, immediately after it was shed, with the embryonic plasma, and when no inhibition of clotting occurred on mixing embryonic plasma with human blood which had remained in contact with glass surfaces for 3 minutes or longer. (6) *Antithrombin*.—This was prepared by heating the embryonic plasma to  $60^{\circ}$  for half an hour; the method of Howell (10) was also used. Its presence was indicated by the restraint of the clotting of both human and embryonic blood after oxalation and recalcification.

### *Results of Experiments.*

*Embryos aged 11 days.*—Negative results were obtained with the tests for fibrinogen, prothrombin and thrombin. Both the whole blood and the plasma remained fluid indefinitely at room temperatures ( $14^{\circ}$ – $17^{\circ}$ ), and at  $40^{\circ}$ . The plasma was markedly alkaline to litmus, but did not clot on neutralization. It did not coagulate on the addition of calcium chloride. No globulin was found, but the plasma contained protein material which coagulated at  $72^{\circ}$ – $75^{\circ}$  and  $80^{\circ}$  respectively. The protective power against the inception of clotting is variable. For example, the immediate mixing of an equal volume of the embryonic plasma with human blood resulted in the mixture remaining fluid at  $16.5^{\circ}$  for 2 hours. A control experiment showed that the human blood used clotted completely at  $16.5^{\circ}$  in 6 minutes 5 seconds. No suppression of clotting followed the mixing of embryonic plasma with human blood which had been shed on glass for 3 minutes or longer. In another instance, the delay of clotting arising from the immediate mixing of embryonic plasma and human blood was 50 minutes; whilst in a third, the delay was only 3 minutes. These examples are typical of those obtained in a large number of experiments.

*Embryos of various ages from 12 to 13 days.*—Two types of plasma were found. (1) That in which prothrombin and fibrinogen are absent, and (2) plasma containing both these substances. The absence of prothrombin and fibrinogen, however, appears to be rare in 13-day embryos. In no case was there any evidence of the presence of prothrombin in plasmas in which fibrinogen was absent. The plasma was strongly alkaline to litmus, but did not coagulate at room temperatures on neutralization, even when both prothrombin and fibrinogen were present. Addition of a few drops of a 1 per cent. solution of calcium chloride to the plasma containing fibrinogen and prothrombin caused clotting in 3 minutes at  $40^{\circ}$ , and in 5 to 10 minutes at  $16.5^{\circ}$ .

No globulin was found in those plasmas which did not contain fibrinogen. Globulin also appeared to be absent from the serum obtained by clotting the plasma and from the protein left in solution after heating the plasma to  $60^{\circ}$ . A

protein which coagulated at 72°–75° was, however, invariably present. A further precipitation of protein invariably occurred on heating to 80°.

The protective power against the inception of the clotting of human blood decreases with the development of fibrinogen and prothrombin and appears to be either absent or almost absent when the capacity of the plasma to clot as a complete gel is developed.

*Embryos aged 14–20 days.*—The blood and plasmas at this stage of development exhibits all the characteristics of that of the adult bird, except that clotting is hastened by the addition of a few drops of a 1 per cent. solution of calcium chloride. All the constituents found in adult blood are present. Coagulation may occur prior to the agglutination and lysis of thrombocytes, and whilst the leucocytes appear intact. When the embryonic blood was mixed with an equal volume of human blood the speed of clotting of the latter fluid was greatly accelerated, commencement and completion of clotting occurring at room temperatures in 1 to 2 minutes and 2 to 3 minutes respectively. Very rapid lysis of both the bird's thrombocytes and the human platelets occurred. The addition of the embryonic plasma to human blood did not hasten its coagulation.

The serum of the embryonic plasma contained a globulin or a mixture of globulins and other protein material which coagulated at 72°–75° and 80° respectively.

#### *General Results.*

Ionised calcium was present in all plasma which contained fibrinogen and prothrombin. In the plasmas of embryos aged 13 to 19 days a shortage of calcium ions appeared probable, as the addition of a few drops of 0·1 per cent. calcium chloride produced complete clotting in 3 to 5 minutes at room temperatures. At 40° the shortage of calcium ions did not prevent coagulation. Similar indications of a deficiency of calcium ions were not found in the plasma of either the twentieth or twenty-first day embryos or in that of adult fowls.

All the plasmas examined, including those of eleventh day embryos, yielded antithrombic material on heating to 60° for 30 minutes, and on applying Howell's test for antithrombin, which also involves heating to 60°. No evidence of antithrombic action was found in unheated plasmas.

The ground-up tissues of all embryos aged over ten days also yielded antithrombic material on heating to 60° and extracting with 0·9 per cent. sodium chloride. The anti-coagulant obtained by heating both embryonic plasma and tissue differed from the natural material, which is protective against

the inception of blood clotting, as it was stable in contact with glass surfaces at temperatures up to  $80^{\circ}$  and restrained the later stage of coagulation in which thrombin is developed. Neither of these properties was exhibited by the natural material. The tissue detritus and saline extracts of the tissues of embryos aged eleven days and older inaugurated coagulation at room temperatures ( $14^{\circ}$ – $17^{\circ}$ ) when added to those embryonic plasmas in which fibrinogen and prothrombin had developed, but had no visible effect on plasma in which these constituents had not developed. The detritus and tissue extract also hastened the clotting of human blood.

The coagulation power of tissue detritus and extract increased with the ageing of the embryos. For example, nearly twice the amount of tissue extract obtained from an eleventh-day embryo was required to produce coagulation at the same rate as when the extract of a sixteenth-day embryo was used.

#### *Conclusions and Discussion.*

The incoagulability of the blood of early chick embryos is due to the absence of both fibrinogen and prothrombin. The plasma prior to the development of these substances contains proteins which coagulate at  $72^{\circ}$ – $75^{\circ}$  and  $80^{\circ}$  respectively.

Fibrinogen and prothrombin appear to develop simultaneously. In no case was one of these substances found in the absence of the other. Support is thus afforded to the suggestion of Mellanby (11) of the close association of fibrinogen and prothrombin in blood plasma.

The appearance of globulin, other than fibrinogen, occurs comparatively late in development (after the twelfth day). Blood coagulation can take place without the appearance of serum globulin. In the serum of embryos aged 14 to 20 days a globulin was, however, found.

An excess of material which is protective against the inception of clotting exists in the plasma of early embryos. This material appears to be more thermostable than fibrinogen, but does not possess antithrombic properties. An excess of similar material has been described in certain hæmophilic bloods (Pickering and Gladstone (12)). Attention is also directed to the finding, by Hess (13), of a shortage of ionised calcium in the plasma of a non-hereditary but recurrent bleeder. In this respect the abnormal human blood also resembled that of embryos. The present writers have, however, found a normal calcium content in the plasma of several hæmophilics. Unpublished experiments by one of us (J. W. P.) indicate that the plasma of recurrent and non-hereditary bleeders may exhibit no apparent abnormalities, either qualitative or quanti-

tative. A re-classification of the blood of bleeders, on a biochemical basis, appears therefore desirable.

An increase of hydron concentration of the embryonic plasma, produced by its neutralization to litmus, provokes clotting at room temperatures. Hydrogen ions thus appear to behave as a catalyst in the clotting of embryonic plasma in a manner similar to that described by Pickering and Reeves (14) in the case of the plasma of adult birds.

Antithrombins were prepared from embryonic blood and other tissues at the stage of their development when the mother substance of thrombin had not appeared in the plasma. This observation is opposed to the current view that antithrombins are produced by a reaction of the organism in response to the presence of thrombin.

The absence of antithrombic properties in unheated plasma and their appearance only after heating to 60° supports the view, suggested by Pickering and Hewitt (15), that antithrombins are artificial products.

The indications of a deficiency of ionised calcium in the plasma of embryos aged 12 to 19 days and the disappearance of the shortage on the twentieth or twenty-first day after incubation corresponds with the finding of Plimmer and Lowndes (16) of a gradual increase in the calcium content of the developing egg. The adhesion of the allantoic vessels to the shell membrane may account for these changes.

A higher concentration of calcium ions is required to permit clotting at room temperatures than suffices at 40°. It thus appears probable that the impact of a definite number of calcium ions in a definite time interval is an essential factor in the inauguration of blood coagulation.

The inception of clotting of embryonic plasma at room temperatures prior to the lysis and agglutination of thrombocytes indicates that the disintegration of these bodies is not essential for the commencement of clotting. This is concordant with recent observations on the thrombocyte-free plasma of adult birds (Pickering and Reeves (14)).

The acceleration of clotting produced by mixing the blood of embryos aged from 14 to 20 days with human blood appears to be due to the very rapid lysis of thrombocytes, as the mixing of the thrombocyte-free plasma of the bird with human blood does not hasten coagulation.

#### *Summary.*

1. The development of various constituents of the plasma of embryo chicks is described and discussed.



2. An excess of material which is protective against the inception of clotting exists in the plasma of early embryos. This material does not possess antithrombic properties.

3. Attention is directed to certain similarities of the behaviour of embryonic and hæmophilic bloods.

4. Antithrombic material can be prepared by heating embryonic blood to 60° at the stage of development prior to the appearance of prothrombin.

5. The inception of the coagulation of embryonic blood may occur, at room temperatures, prior to the agglutination and lysis of thrombocytes.

(6) The rôle of both hydriens and calcium ions in blood coagulation is discussed.

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*A Critical Study of the Direct Method of Measuring the Osmotic Pressure of Hæmoglobin.*

By G. S. ADAIR.

(Communicated by Sir Wm Hardy, Sec.R.S.—Received April 1, 1925 )

*Summary.\**

A brief account is given of the causes of variations in osmometer readings, and experimental methods and sources of error are discussed.

Particular attention is given to the criteria which may serve to distinguish true osmotic pressures from temporary diffusion pressures and false equilibria.

Experimental proof is given that, in certain solvents, the osmometric observations on hæmoglobin satisfy the three criteria—permanence, reversibility and reproducibility ; therefore they may be regarded as true osmotic pressures.

Readings remained constant within 6 per cent. for nine weeks, and no products of protein breakdown could be detected in the outer liquids.

With rigorous control of conditions and with adequate criteria for equilibrium, the osmometer method proved thoroughly reliable ; and when certain corrections were applied, it proved capable of giving results accurate to 0·1 mm. of mercury, which corresponds to about 1/100.000th of a degree in depression of the freezing point.

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\* The full paper is printed at ' Proceedings,' Series A, vol. 108, pp. 627-637 (August, 1925).

*The Osmotic Pressure of Hæmoglobin in the Absence of Salts.*

By G. S. ADAIR.

(Communicated by Sir Wm. Hardy, Sec.R.S.—Received April 1, 1925.)

*Summary.\**

The osmotic pressure determinations for dialysed hæmoglobin made by previous workers ranged from 3·5 mm. to 12·1 mm. per 1 per cent. of protein.

Variation is to be expected, for it is theoretically impossible to prepare a hæmoglobin solution absolutely free from combined acids or bases, and there is the further risk that prolonged purification may cause changes in the protein.

The theory formerly accepted was that the high pressures (about 10 mm.) represented pure hæmoglobin of molecular weight 16,700, the same as the equivalent determined by iron analyses. The lower pressures were attributed to aggregations of the molecules caused by salts.

Repetition of the work with iso-electric hæmoglobin gave pressures of 3·2 mm., or less, and pressures of 10 mm. or more were obtained only when conductivity and hydrogen-ion determinations showed that acid or base was bound.

The determinations of Hüfner (10 mm.) were made at an unknown hydrogen-ion concentration, and it is here suggested that most of the pressure in his experiments was due to undialysed acid or base. The molecular weight of pure hæmoglobin is about four times the equivalent.

\* The full paper is printed at 'Proceedings,' Series A, vol. 109, pp. 292-300 (October, 1925).

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*Studies on the Nature of the Immunity Reaction.—I. An Experimental Study of Pneumococcal Immunity.*

By RICHARD R. ARMSTRONG, M.D., M.R.C.P.

(Communicated by Sir Frederick Andrewes, F.R.S.—Received, April 8, 1925.)

Pneumococcal infection, both in man and animals, has been the subject of study by the writer during several years past. A careful histological investigation of the morbid histology of pneumonia, carried out in conjunction with Gaskell (1), in 1913-14, using his method of gelatin-embedded sections, led to the conclusion that infection of the lungs, in this disease, is by way of the air passages and not, as formerly believed, by the blood stream. Experimental proof of this view was afforded by the successful production of pneumonia in rabbits by intratracheal insufflation (2) (1914). The existence, in London, of serological types of pneumococcus, identical with those found in America and South Africa, was demonstrated (3) in 1919-20. This work was extended, the year afterwards, with the aid of the "absorption of agglutinin" test, to the serological study of the pneumococcus group, various sub-types of the recognised disease-producing strains were then identified (4). A study of the means by which immunity from pneumococcal infection is acquired was a natural sequence to these researches.

It is already well known that inoculation of laboratory animals, rabbits and mice, with killed cultures of disease-producing pneumococci, renders these animals proof against subsequent infection with living organisms of the same strain. Furthermore, it has been found that the blood serum of an artificially immunised rabbit and that of the human patient after crisis and recovery from lobar pneumonia equally possess immunising properties; when inoculated into the naturally susceptible mouse, these sera confer protection against subsequent infection with living pneumococcus of the homologous strain.

A quantitative estimation of the progress of the immunity following inoculation or natural infection seemed necessary. The essential feature of the method devised was that it involved the titration of the potency of protective sera in terms of the living pneumococcus, using mice as test objects.

Consistent results were obtained only when certain experimental conditions were satisfied. The factors controlling the maintenance, at constant virulence, of the strain of Type II pneumococcus selected were first ascertained. Once standard culture virulence was established, the determination of the minimal

lethal dose for mice became a simple matter. Finally, 0.5 c.c. of a broth dilution containing one-millionth of 1 c.c. of blood broth culture, not more than 16 hours old, was adopted as a standard minimal lethal dose. In the experiments described, a minimal dose (0.2 c.c.) of serum contained sufficient immunising substance to neutralise one minimal lethal dose of pneumococci.

Mice of 18-22 grammes in weight are exceedingly delicate indicators of the protective power of immune sera. The animals were inoculated intraperitoneally with 0.2 c.c. of the serum under test. At the same time a culture was made in blood broth of the strain of Type II pneumococcus of standard virulence and incubated at 37° C. Sixteen hours later, conveniently on the following morning, decimal dilutions of this broth culture were made in tubes each containing 9 c.c. of warm broth. The prepared mice then received, intraperitoneally as before, 0.5 c.c. of broth dilutions of known strength. When the potency of the sample was insufficient to protect against a particular dilution of the culture, the mouse died after from two to five days. It was possible in this way not only to titrate a range of protective power from 1 to 200,000 minimal protective doses but even to detect the subtle differences between unit protective power and complete absence of protection, first, by the slower onset of the symptoms of illness; secondly, by its more prolonged course than in the control animals which had not been inoculated with serum. In this way, it was discovered that the serum of a healthy rabbit has slight protective power for mice against the pneumococcus. Although mice inoculated with 0.5 c.c. rabbit's normal serum mostly succumb, animals receiving 1 c.c. usually survive an intraperitoneal inoculation of one minimal lethal dose of Type II pneumococcus administered 16 hours later. Human normal serum confers no protection on mice against the pneumococcus.

*Immunity following Single Dose Inoculation*—In each experiment, a rabbit was inoculated intravenously with a single large dose of 50,000 million killed pneumococcus vaccine. Samples of blood were then collected daily, the sera separated and stored on ice. At the end of each experiment, the batch of samples was tested for protective substances. Chart 1 is a graphic representation of the results of a typical experiment.

It is impossible to represent, in arithmetic progression, on a single chart, a range of serum potency varying from 1 to 200,000 minimal protective doses. The practice of decimal dilution of pneumococcal cultures is, however, directly adaptable to the expression of the numerical results on a logarithmic scale. Accordingly, in all diagrams, the figures given in the left margin are the characteristics of the logarithms of the number of minimal protective units in

standard doses of 0.2 c.c. of serum : the protective power of serum samples, at successive intervals of time, whether deduced from inoculated rabbits or

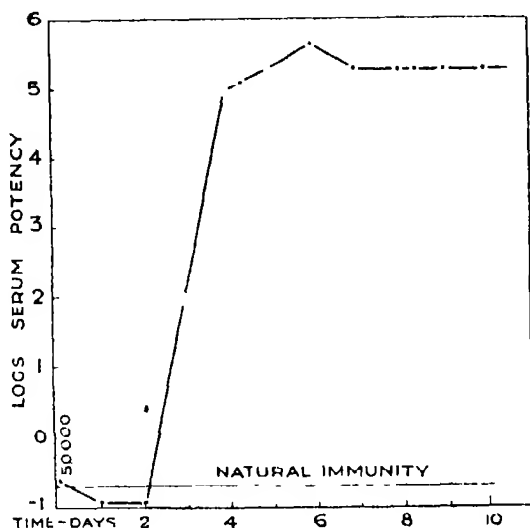


CHART I \*—Immunity response in rabbit following intravenous inoculation of a single large dose of vaccine. (Doses throughout are quoted in millions)

cases of pneumonia, is charted as abscissæ, the time in hours and days as ordinates. It is an inherent defect of the use of a logarithmic scale that small differences in protective power at the bottom of the scale are disproportionately enhanced in graphic representations, whilst large differences at the summit of the curve are minimised.

It will be noted that the first event following inoculation is the disappearance from the serum of all traces of natural protective power. This effect persists during two days in exceptionally large and vigorous animals and in the average laboratory rabbit over three days. The interval may well be spoken of as an "inductive period". At the expiration of the period, the serum becomes protective with great suddenness and rapidly increases in protective power, the rate of increase being represented graphically by a straight line, presumably indicating a geometric rate of production of the protective anti-bodies. This abrupt linear rise persists during two days, after which, although the potency

\* In this and subsequent diagrams serum potency is expressed as the logs of the numbers of minimal protective doses for mice in samples of 0.2 c.c. of serum. The figures in left-hand margin are the logarithmic characteristics: hence 0 represents 1 minimal protective dose; 6, one million doses.

of the serum continues to increase, the rise is at a more gradual rate, reaching a maximum on the sixth day after inoculation. A slight fall from the maximum titre is followed by the persistence of protective power at a constant high level.

The shape of the immunity curve and the attainment of a high degree of immunity on the sixth day vividly recalls the occurrence of crisis in pneumonia at about this time and may well afford an explanation of the phenomenon.

The immunity following repeated increasing doses of vaccine (Chart 2) is strikingly similar to that which follows inoculation of a single dose. This effect was studied experimentally by intravenously inoculating rabbits with increasing doses of vaccine at intervals of 48 hours, so as roughly to imitate the natural increase of the pneumococcal population of the lung in pneumonia.

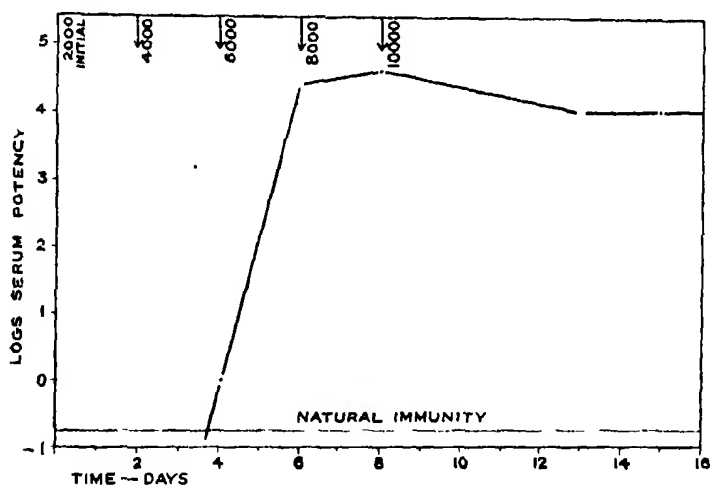


CHART 2.—Development of immunity in serum of rabbit following increasing doses of vaccine.

The maximum level of immunity attained is rather less than that shown in Chart 1. Slight variations in the character of the immunity response between one experiment and another will be noted on comparison of many of the charts that follow. These variations are due to slight differences in the activity of different batches of vaccine and in the response to inoculation of different rabbits. It is found, however, that a selected batch of the animals in good health and of the same weight, age and breed, inoculated with the same batch of vaccine and kept under identical conditions, give results which are *inter se* consistent and comparable. Experimental control animals were used whenever comparative tests were made.

Chart 3 displays the clinical and serological picture in a representative case of lobar pneumonia in an adult man. The development of serum immunity

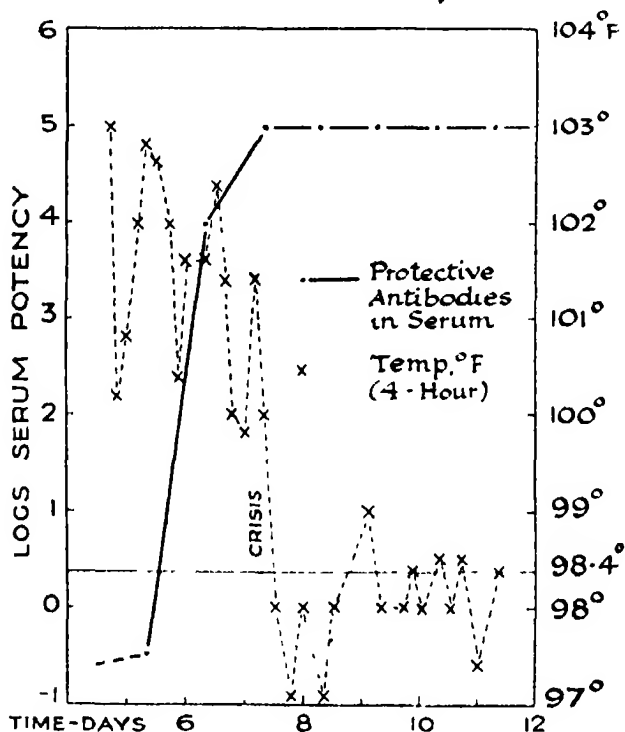


CHART 3.—Human lobar pneumonia.

is contrasted with the progress of the illness, as recorded by four-hourly temperature readings.

An "inductive" period of four or five days follows the rigor which usually marks the onset of pneumonia in a typical case. Numerous experimental tests have proved that normal human serum has no natural protective power against the pneumococcus, such as is found in rabbit's serum.

Protective power first develops on the fifth or sixth day and increases rapidly during a day or two. The curve shows an abrupt linear rise, very similar to that observed in the inoculated rabbit. The increase then proceeds at a slower rate, to a maximum on the eighth or ninth day, on which day the critical fall in temperature, with striking amelioration in the patient's condition, known as "crisis," usually takes place.

The appearance of protective anti-bodies in the blood serum is a characteristic phenomenon in the response of the animal to pneumococcal infection. That



these are not formed by the action of pneumococcal antigen on any constituent of the circulating blood follows from the fact that the protective substances cannot be obtained by the cultivation of pneumococci in sera *in vitro*, nor are they present as a result of the multiplication of pneumococci in the blood-stream *in vivo* in cases of septicæmia.

It is probable, therefore, that the seat of formation of the anti-pneumococcal substance is located in the tissues, very possibly in cells specially concerned with the defence of the body against bacteria. Hence it is conceivable that tissue immunity may develop before a sufficient excess of protective substance is produced to appear in the circulating blood. In such a case, vaccinated animals might prove resistant to infection before the development of serum-immunity.

To test this hypothesis, a large number of mice were simultaneously inoculated intraperitoneally with equal doses of the same batch of killed pneumococcal emulsion. Day by day thereafter a batch of animals was selected and their immunity tested by intraperitoneal inoculation, a range of doses of the living homologous pneumococcus being given. Blank control animals were simultaneously inoculated each day with a minimal lethal dose of the culture used. Survival or death of adjacent numbers of a series indicated the degree of immunity attained.

The pooled results of three separate experiments, each confirmatory of the other, are depicted graphically in Chart 4. A very large number of mice, more than 150, had to be tested before sufficient satisfactory daily estimations of immunity could be obtained. The day of appearance of immunity and the abrupt rise in the power of the animals to resist infection, to a maximum on the sixth day after inoculation, coincide almost exactly with the course of events in the serum of the vaccinated rabbit. There is good reason to believe, therefore, that protective anti-bodies appear in the serum as soon as formed, and that their amount is a faithful index of the degree of resistance to infection of the animal as a whole.

The rather rapid fall of immunity after the sixth day, in comparison with that shown in Chart 1, has formed the subject of a separate inquiry. It is believed that it was a consequence of the use of the peritoneal cavity instead of the blood stream as the route for prophylactic inoculation.

*Summary.*—A comparison of the immunity in response either to a single inoculation of pneumococcal vaccine administered intravenously or intraperitoneally, or to repeated increasing doses given intravenously, with that which follows the persistently increasing stimulus of the rapidly multiplying

pneumococci in the lung in human lobar pneumonia, brings to light a striking similarity in the shape of the immunity curve in all three conditions.

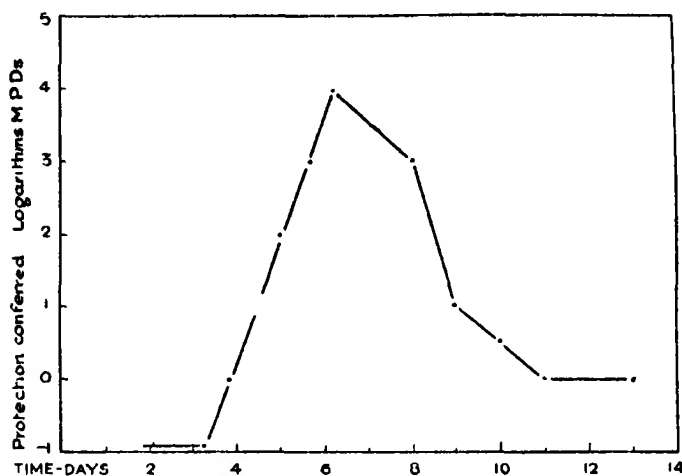


CHART 4.—Immunity conferred on mice by single dose of 10,000 million type II pneumococcus vaccine.

The chief outcome of the rigidly quantitative study of pneumococcal infection described has been the discovery that immunity is not continuously and gradually developed from the moment when the antigenic stimulus is first applied. The pneumococcal vaccine apparently excites some definite orderly change, lasting over two or three days, and it is not until the end of this time that a specific protective material is poured out into the blood-stream at a rapid geometric rate.

A further interesting observation is the proof afforded by the quantitative study that normal rabbit's serum possesses slight but definite anti-infectious power, and that the first result of prophylactic inoculation is the disappearance of this natural immunity which constitutes the "negative phase" so much dreaded by vaccine therapists. The comparatively long duration of the inductive phase is less easily explained. The delay is possibly, in part, due to the slowness of the preliminary interaction of the bacterial bodies and the defensive mechanism of the host.

Each aspect of the subject calls for further experimental study. In the first instance, it will be necessary to inquire if any correlation exist between size of dose and intensity of response. Secondly, it will be well to ascertain how far the mechanism of immunity is susceptible to further stimulus during the inductive period. Thirdly, it seems expedient to study factors likely to

influence the phases of rising immunity and of established immunity, for such an investigation may well assist in the interpretation of the spread of infection and of relapse in lobar pneumonia.

*Relation between Size of Vaccine Dose and Immunity Response.*—In all the experiments described hitherto, large doses of vaccine, of the order of 10,000 to 50,000 million organisms, were administered. These doses were chosen arbitrarily and far exceed the limits of dosage commonly used in vaccination of the human subject. It is noteworthy, however, in this connection, that Lister has shown (5) that lasting immunity could only be conferred on Kaffirs and on himself by giving very large doses, and that Cecil and Steffen (6) found that doses of the order of 60,000 million cocci were required to protect monkeys against experimentally produced pneumonia.

Chart 5 summarises the results of the intravenous inoculation of rabbits with single doses of killed pneumococcal vaccine ranging from 500 organisms to 500,000 million organisms. The experiment was repeated three times with different batches of rabbits. The pooled results are shown.

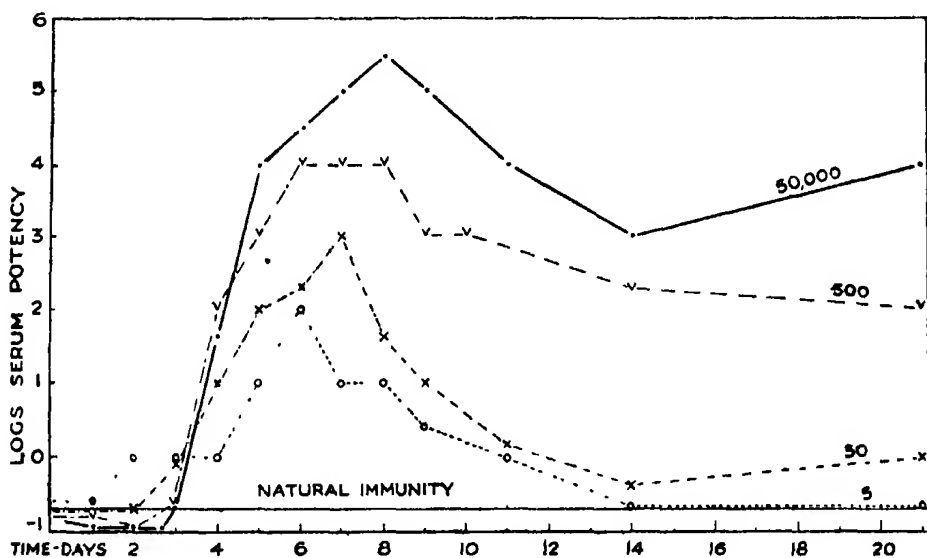


CHART 5—Correlation between size of vaccine dose and immunity response.  
Doses in millions.

The smallest doses of vaccine, 500 organisms and 5,000 organisms, had no noticeable antigenic effect, nor was experimental evidence obtained of any neutralisation of natural immunity following inoculation. It is tempting to suggest that small doses of killed organisms are dealt with by the natural

non-specific protective mechanism of the blood, to which reference has already been made (p. 2), so that there is never sufficient antigen available to excite the production of anti-bodies. The minimal effective dose of antigen required to set the immunising mechanism in operation appears to be of the order of 50,000 organisms ; traces of protective power were apparent on the sixth to the seventh days after this dose. A good and proportionate yield of the immunising substance followed the administration of larger doses, up to 50,000 million organisms, the persistence of immunity being more prolonged the higher the dose. The effect of administration of an excessive dose, 500,000 million organisms, is of great interest. Even on the seventh day after inoculation, the presence of free protective substance could hardly be detected. None the less, the natural powers of response eventually triumphed, for on the fourteenth day the degree of serum immunity nearly equalled that following a dose of 50,000 million cocci and continued to march *pari passu* with it until at least a week later (Chart 10). It is easy to picture the condition of a case of lobar pneumonia suffering from a similar overwhelming infection and to explain the occurrence of death before the immunising mechanism can rally.

A striking proof of the correlation between dose and response is afforded by Chart 5. Attention is directed to the following significant points ---

(1) The smaller the dose of vaccine, the less complete and the shorter is the phase of neutralisation.

(2) The smaller the dose the earlier does immunity appear, and the sooner is maximum immunity attained.

(3) The smaller the dose the shorter is the period during which immunity persists.

(4) The maximum immunity attained is proportional to the size of the vaccine dose, provided the dose be not too large.

The drop in the immunity curves on the 14th day and subsequent rise is probably due to the fact that the concentration of protective substances in the serum is greatly lowered by the daily bleedings in rabbits of moderate size—1,000–1,400 grams. Lines joining the curve maxima with the records on the 21st day probably more nearly represent the rate of fall of the titre, this rate is more rapid, the smaller the dose of vaccine given.

The maximum concentration of protective anti bodies on any one day is plotted against the size of vaccine dose in Chart 6. This chart illustrates even more clearly than Chart 5 how close is the proportionate relationship between the size of the dose of pneumococcal vaccine and the maximum immunity developed in response thereto.

It will be noted that the immunity curve follows a straight line until doses over 500 million are reached, after which the concentration of protective substances does not keep pace with the increase of the dose of vaccine.

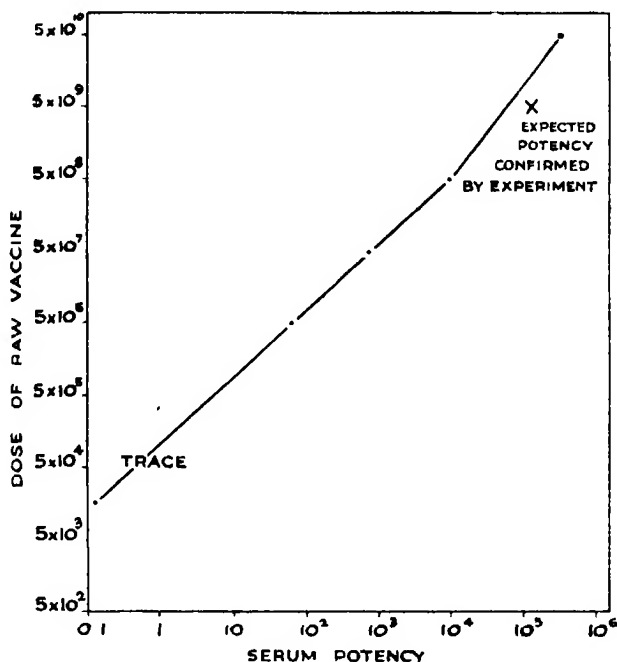


CHART 6.—Maximum proportionate increase of serum-immunity with vaccine dose.

There is good reason to believe that this departure from the theoretical value is due to the fact that a concentration of 200,000 minimal protective doses of the protective substances per 0.2 c.c. of serum nearly represents the saturation point of rabbit's serum, above which the excess is probably excreted.

*The Optimum Dose of Pneumococcal Vaccine.*—Reference may conveniently be made at this point to the experimental evidence on which is based the suggestion that the serum of immunised rabbits may become saturated with the protective agent.

It is as follows : no sample of serum from any of the large number of rabbits immunised in the course of the inquiry has at any time exceeded a titre of 500,000 minimal protective doses per standard serum volume 0.2 c.c. ; in the great majority of instances, 200,000 doses per 0.2 c.c. serum has represented the maximum potency attained. In addition, the immunity curve remains a flat plateau during many days, in the case of large vigorous rabbits, after the maximum degree of immunity is reached on the sixth day.

The results shown graphically in Chart 5 strongly suggest that, in the case of large doses of vaccine, the output of immunising substances does not reach a maximum till about the eighth day after inoculation, a fact not without significance in view of the occurrence of crisis in lobar pneumonia on the eighth day. It seems not improbable, therefore, that the plateaux depicted in Charts 1, 7 and 9 indicate serum saturation levels in the particular animals used. Excess production on the eighth day has been masked by excretion of the surplus material.

From a theoretical standpoint, therefore, a dose of 50,000 million pneumococci is in excess of that required to exert a sufficient antigenic stimulus to raise the concentration of protective substances in the circulating blood of the rabbit to saturation point. The dose just sufficient to excite saturation may therefore be regarded as the optimum dose. Whilst it does not necessarily follow that 50,000 million organisms represent a bulk of antigen in excess of the maximum capacity of the mechanism of the rabbit by which the protective anti-bodies are formed, there can be no doubt on experimental grounds that a dose of 500,000 million organisms is vastly beyond this capacity, there seems to be no alternative explanation of the long postponement of serum immunity following this dose.

These deductions were put to practical test by the inoculation of a rabbit with 5,000 million organisms. A maximum immunity of 100,000 minimal protective doses per 0.2 c.c. serum was reached on the ninth day and rapidly fell thereafter. This titre corresponds to the theoretical maximum for the dose deduced from Chart 6. A study of the Chart suggests that the presumed saturation yield of 200,000 minimal protective doses should be given in response to a dose of the order of 10,000 million organisms.

The conception of the existence of such a saturation level may well have important bearing on the conditions limiting the production of high titre immunising sera for therapeutic purposes. For the reasons advanced it seems physically impossible to prepare sera of more than a certain potency, determined by the saturation point. If this be so, the effective use of pneumococcal serum in manageable doses seems seriously restricted.

*The Immunity Response during the Phase of Induction.*—Granted that there is a close analogy between the immunity response following administration of vaccine, and that in a favourable case of lobar pneumonia, it becomes of great importance to ascertain how far the mechanism of immunity is susceptible to further stimulus during the phase of induction, before immunity develops, for it is at this time, if at all, that specific therapeutic measures are likely to be of value in pneumonia.

Chart 7 shows the result of administering intravenously to a rabbit doses of 15,000 million and 25,000 million pneumococci at intervals of 24 and 48 hours after an initial dose of 10,000 million pneumococci.

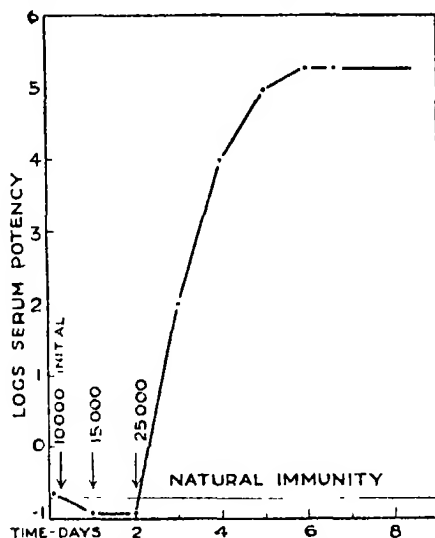


CHART 7.—Vaccination daily during inductive phase.

It is significant that no postponement and little, if any, alteration in shape of the logarithmic phase of immunity followed even so large a dose as 25,000 million cocci. The result should be compared with that in Chart 1, which served as a control for this experiment; also with that depicted in Chart 9. The hypothetical optimum dose, for the full stimulation of the antigenic mechanism in the rabbit, being an initial dose of 10,000 million, the apparent absence of any alteration of the immunity curve, after inoculation of further large doses of vaccine, might have been a consequence of the fact that the protecting mechanism was fully occupied and hence insusceptible. In order to test this hypothesis, a small dose of 50 million cocci was administered intravenously to a rabbit, and followed in 48 hours by a much larger dose of 50,000 million cocci. The appearance of immunity was postponed slightly by this procedure, but the maximum immunity developed was much greater than in the case of the control animal, which received 50 million cocci only (Chart 8).

It appears, therefore, that the rabbit is susceptible to further stimulation during the inductive period following a dose of vaccine, provided the initial dose has been insufficient to appropriate all the means available for the production of protective anti-bodies.

It will be noted that the total immunity attained by the animal which received 50 million followed by 50,000 million cocci is less than might

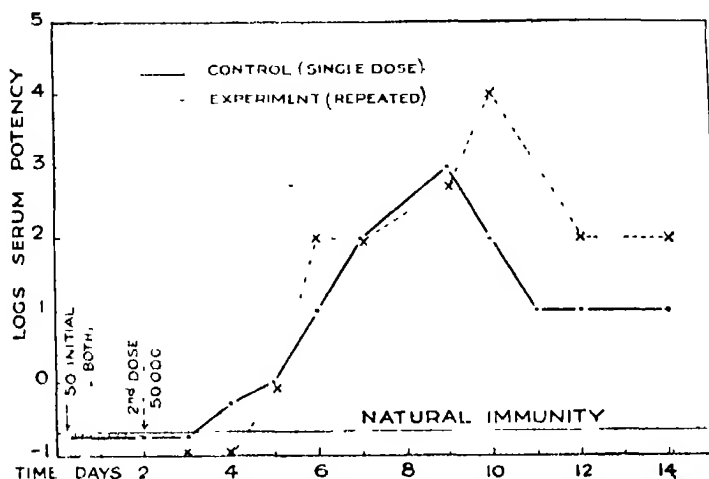


CHART 8.—Vaccination during inductive phase, 48 hours after initial dose.

have been expected had the animal received 50,000 million cocci in a single dose. This apparent anomaly has been investigated, and it is believed satisfactorily explained by the results of experiments to be described elsewhere (Part II). The main result remains unchallenged, namely, that, provided the initial dose of vaccine administered be less than the "optimum" dose, the protecting mechanism is capable of responding to further stimulus during the inductive phase.

*The Immunity Reaction during the Phases of Rising and Established Immunity.*

—A rapid fall in immunity occurs if an initial large dose of vaccine be followed on the fourth day by a second large dose. At this stage in the immunity reaction the concentration of anti-bodies is below the maximum (Chart 9). Twenty-four hours later protective power has completely disappeared. Nevertheless a high titre is regained in 48 hours and persists. The neutralisation of immunity apparently takes some time to accomplish, and is by no means complete (about one-third neutralisation) after an interval of eight hours.

A second dose of vaccine, 50,000 million cocci, administered on the eighth day, when the output of anti-bodies was at a maximum, proved insufficient to disturb the titre of serum samples collected eight hours later and subsequently.

In another experiment (Chart 10) an initial dose of 50,000 million cocci was administered to a rabbit which responded in an orthodox manner.



A massive dose of 500,000 million cocci was simultaneously administered to a companion animal. A prolonged phase of neutralisation followed, lasting

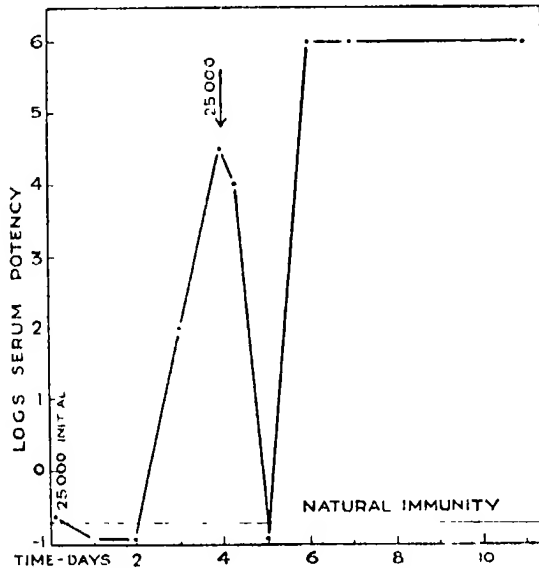


CHART 9—Effect of vaccination during rising immunity.

six days. During the next five days a protective power of no more than one minimal lethal dose per standard volume (0.2 c.c.) of serum could be demonstrated. Nevertheless 14 days after inoculation the titre had risen to 1,000 doses per 0.2 c.c. of serum, and on the 21st day the titre of the serum of both animals was the same. At this point a dose of 50,000 million cocci was administered to the animal which had received the massive dose, to test particularly whether the power of response of the protecting mechanism was impaired by the excessive dose of vaccine.

As shown on the chart, the immunity fell but 48 hours later the serum concentration had returned to its former level; five days later an increase in serum potency became apparent; on the seventh day the serum immunity was at a maximum. Clearly, therefore, overdose had not destroyed the power of the animal to respond to a maximal extent to further stimulus.

Both the rabbit and its control were probably in an identical stage of immunity on the 21st day, the curve of serum immunity having been so nearly parallel during seven days. A colossal dose—two and a half billion cocci—was given intravenously to the control animal on this day, with the result that immunity was completely lacking during fully 24 hours. Despite this rude shock, the

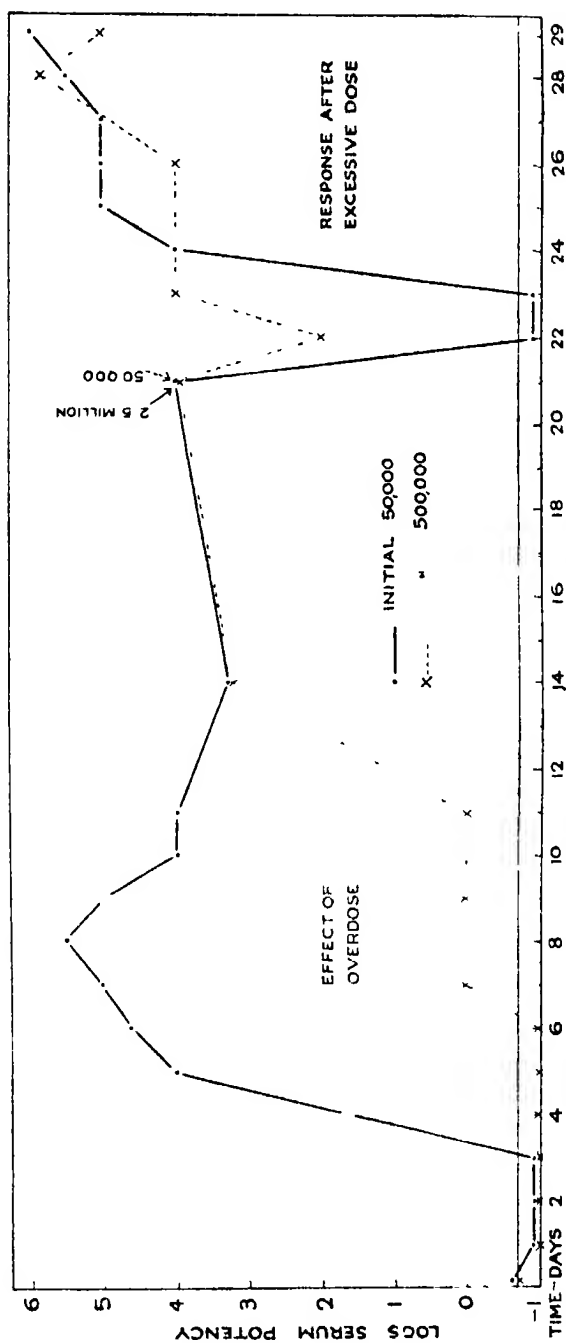


CHART 10 — Response after excessive dose of vaccine

protecting mechanism suffered no damage and responded to the maximum extent a few days later.

The experiment furnishes an explanation of the extreme rarity of relapse in pneumonia after crisis has once occurred. Not only does the active immunity persist sufficiently long to render fresh infection extremely unlikely ; even if a fresh infection should potentially arise long enough after crisis for immunity to have fallen to a low level, an efficient mechanism is available to support this residual immunity in repelling the invader. Reinfection under these conditions becomes practically impossible.

[*Note added July 20.*—My attention has been called to the experiments of Henderson-Smith and St. John Brooks (7) on the effects of dosage in the typhoid vaccination of rabbits. Batches of rabbits were inoculated subcutaneously with a range of doses of a standardised suspension of heat-killed typhoid vaccine, respectively containing 20-, 100-, 500-, 2,500- and 12,500-million organisms. Day by day, thereafter, small samples of blood were taken, the separated sera pooled and tested *in vitro* for agglutinating power, complement deviation, precipitin formation, opsonic power and bactericidal power.

The results obtained closely parallel those described above, dealing with the gross protective power *in vivo* of the sera of rabbits after inoculation with pneumococcal vaccine. With the exception of bactericidal action, specific anti-bodies against the typhoid bacillus appeared at about the fifth day, the response being in every case proportionate to the dose administered ; the larger the dose the later was the time of attainment of a maximum titre, up to the 10th-11th day.

These authors note that the response proportionately lessens with increasing vaccine dose and anticipate but do not demonstrate a limiting value. (*Cf.* Chart 6 above )

But whereas in the case of the pneumococcus, the " inductive " or " negative " phase varies in intensity and duration directly with the size of vaccine dose, so that the superimposed curves successively intersect (*v* Chart 5 above) ; in the experiments on typhoid vaccine no such variation was found, the anti-bodies studied appearing at about the same time after inoculation, irrespective of dose, so that the curves were simply superimposed. A possible explanation of this difference lies in the fact that, in the writer's experiments, a method was available for estimating the approximate intensity of the " negative " phase, and that in the one case immunisation was effected intravenously and in the other subcutaneously.

That the immunity response to the pneumococcus differs in no material

respect from that following inoculation of any other bacterial antigen is apparent also from study of the anti-toxin curve after administration of a single dose of diphtheric toxin (Ehrlich and Brieger ; Salomonsen and Madsen), the phases of which are identical with those following a single dose of pneumococcal vaccine.

The late G. Dean, in an article in Nuttall and Graham-Smith's ' Monograph on the Bacteriology of Diphtheria ' (8), describes the results of experiments in which he found that to obtain an optimum yield of anti-toxin .—

- (1) Pure toxin alone should be used.
- (2) The initial doses given should be small.
- (3) Inoculation should invariably be made during the negative phase.
- (4) The increase in dose should be gradual.

Using this method, not only was a serum of very high anti-toxic potency obtained at the end of seven weeks, but the curve of increase of anti-toxic power showed a regular, rapid, uninterrupted rise throughout the period. Similar conclusions must be drawn from the experiments described in the latter part of this paper.

The favourable effect of summation of increasing sub-maximal stimuli applied during successive " inductive " phases, in the case of the pneumococcus, is well shown in Chart 11

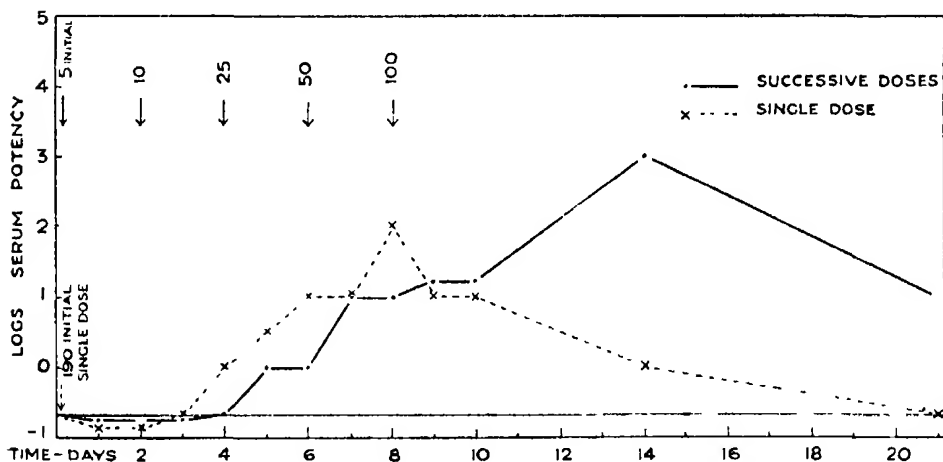


CHART 11.—Typical (human) vaccine course compared with effect of single total dose.

The result of administration intravenously to a rabbit of a series of doses of vaccine of the same order as those commonly employed subcutaneously in therapeutic inoculation of the human subject is here contrasted with the inoculation of a companion animal with a single initial dose, equalling the sum

of the successive doses. Maximum immunity is attained later after successive dosage, but reaches a higher level and persists longer.

A study of Dean's diphtheric anti-toxin curves suggests that there is far greater scope for the production of anti-toxin than for anti-bacterial sera of the antipneumococcal class. A single dose of pneumococcal vaccine of the order of 10,000 million organisms suffices to elicit the production, within a period of a few days, of a high titre serum in the rabbit. There is little, if any, constitutional disturbance of the animal immunised, for the pneumococcal antigen is relatively innocuous. Yet, unfortunately, the maximum anti-body titre obtainable is, from a therapeutic standpoint, ineffective.

On the other hand, it is impossible to administer the optimum initial dose of diphtheric toxin to produce rapidly a high serum concentration of antitoxin, owing to the extremely poisonous effects of the toxin. Hence a progressive slow immunization, taking weeks to complete, is necessary ; at the end of this time, however, an anti-toxic serum is produced on a par, in its curative power, with the extreme toxicity of the diphtheritic toxin.

Apart from these differences, which, however, rigidly determine the conditions of experimental and therefore of therapeutic production of anti-sera, the mode of immunity response to the several antigens seems the same ]

### *Conclusion.*

Quantitative measurements of the amount of protective substance present in the blood serum of animals and men under varying conditions of experimental inoculation or infection with pneumococcus have been made which show :—

1. That experimental inoculation or natural infection with pneumococcus is followed by a definite orderly sequence of events culminating in the development of immunity.

2. That the development of immunity may be expressed graphically by a logarithmic curve having the following characteristics :—

- (1) A phase of neutralisation of natural non-specific immunity which may be described as the Inductive Period.

- (2) A phase of rapidly developing and increasing immunity, which may be described as the period of abrupt linear (logarithmic) rise, during which the amount of protective anti-bodies increases in a geometric proportion.

- (3) A phase during which the immunity rises regularly but more slowly to a maximum.

(4) A phase of constant high immunity, which may be regarded as the period during which, probably, the serum is saturated with immunising substances.

3. That resistance of an immunised animal to infection with the homologous living pneumococcus coincides with the appearance of a protective substance in the blood serum.

4. That, provided very large or very small doses of vaccine be avoided, an exact correlation exists between the size of a dose of pneumococcus vaccine inoculated and the immunity response. The following points are of importance :—

(1) The smaller the vaccine dose, the less complete and shorter is the phase of neutralisation and induction.

(2) The smaller the dose the earlier does immunity appear and the sooner is maximum immunity attained

(3) The smaller the dose the shorter is the period during which active immunity persists

(4) The maximum immunity attained is proportional to the size of the dose of vaccine, provided this be not too large.

5. That small doses of vaccine, from 500 to 5,000 organisms, appear to exert no antigenic effect in the rabbit. It is suggested that these small numbers of bacteria are dealt with by the natural non-specific mechanisms of the body.

6. That excessive doses of vaccine cause prolonged postponement of immunity. Nevertheless, protective anti-bodies eventually appear in the serum. The power of response to further vaccine dosage is unimpaired, provided recovery from the initial excessive dose has taken place.

7. That the optimum dose of Type II pneumococcus vaccine is approximately 10,000 million organisms. This dose just suffices to determine the formation of sufficient protective anti-bodies to saturate the rabbit's serum

8. That the protecting mechanism of the rabbit is proportionately susceptible to additional stimulus during the inductive phase following vaccination, provided the initial dose of vaccine be less than an optimum dose.

9. That anti-bodies may be temporarily removed from the circulating blood, both during the phases of rising and of established immunity, provided that the rate of production of protective substances during a given time be less than the dose of vaccine inoculated.

*Summary.*

It has been found possible to measure, with a high degree of accuracy, the protective power of sera, prepared by immunising rabbits with pneumococcal vaccine, against measured doses of living pneumococcal cultures, using mice as test objects. Immunity is developed in a very remarkable and characteristic manner whether it be following experimental inoculation in the rabbit or the natural stimulus of a lobar pneumonia in man. An inductive phase, lasting from three to five days, is followed by an out-pouring of protective anti-bodies into the serum at a rapidly increasing rate corresponding to a geometric progression, a maximum concentration of protective substance being reached on the eighth day after inoculation. Strict correlation is found to exist, within certain limits, between the size of the dose of vaccine administered and the degree of immunity developed. The influence of further dosage of vaccine during the inductive phase and the phases of rising and established immunity has been studied, together with the effects of overdose on the immunity response.

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*Studies on the Nature of the Immunity Reaction.—II. A Comparison of the Antigenic Properties of Sensitised and Raw Pneumococcal Vaccines.*

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(Communicated by Sir Frederick Andrewes, F R S —Received April 8, 1925 )

THE opinion is current that "Sensitised Vaccines," prepared by saturating living or dead bacteria with the homologous immunising serum, afford a convenient means of administering protective anti-bodies. The use of such vaccines, in moderate doses, is apparently unattended by any harmful local or general reaction.

Two hypotheses have been formulated to explain the mechanism of their action. Besredka (1) and also Gordon (2) lay stress on the fact that sensitisation accelerates the ingestion of bacteria by phagocytes and their consequent intra-cellular digestion ; as a result, endotoxin is liberated and an anti-endotoxin rapidly produced by the phagocytes. Active immunity is thus attained more swiftly than when a raw vaccine, relatively resistant to phagocytosis, is administered.

An alternative hypothesis, which Huntoon and his colleagues (3) have based upon experiments with sensitised pneumococcal vaccines, is that the swift action of sensitised vaccines arises from the rapid liberation of the neutralising charge which they carry and the immediate production of an immunity comparable with that following inoculation of protective serum. The bacterial residue then exerts a deferred influence as an excitant of an immunity similar to that given by raw vaccine.

The objective of the present inquiry has been a comparative study, by quantitative methods, of the properties of sensitised and raw pneumococcal vaccines, with a view to determine the influence of sensitisation. There is no exact information on this subject, as only qualitative tests of the antigenic value of sensitised as compared with raw vaccines have been made. Such clinical evidence as is available is uncontrolled, and consequently of uncertain scientific value. Conclusions as to the efficacy of sensitised vaccines in disease have been based upon the absence of unfavourable effects following inoculation, and on the favourable course of illnesses, which might, in any event, have terminated in recovery.



*Preparation of Sensitised Pneumococcal Vaccine.*

A sensitised pneumococcal vaccine was prepared as follows. Virulent cultures, recently activated by passage through mice, were grown, during at most 16 hours, on Trypagar containing a little fresh blood. The growth was washed and scraped off the medium and centrifugalsed at slow speed, to remove particles of agar and clumps of adherent cocci. The uniform supernatant emulsion was then poured off and its strength estimated by an opacity test. Equal volumes of an emulsion containing 50,000 million cocci per cubic centimetre and of an homologous serum capable of causing agglutination in a dilution of 1 : 80, diluted in the proportion of 1 in 5 with normal saline, were mixed in a centrifugal tube and incubated in the water bath at 37° C. during two hours. It was found to be desirable to shake or stir up the cocci frequently to prevent the formation of a firm agglutinate. The thus sensitised vaccine was centrifugalsed and the exhausted serum rejected; the vaccine was then re-suspended in 0.85 per cent. salt solution, and again precipitated by centrifugalisation, the washing being repeated five times to ensure complete removal of serum. Centrifugalisation tends to cause firm coagulation of sensitised pneumococci, the coagulum may conveniently be broken up, at a later stage, by repeatedly drawing the coarse suspension through a fine-bored needle, attached to an ordinary glass syringe. It is impossible accurately to measure the strength of a sensitised vaccine; this must be computed from the strength and quantity of the pneumococcal emulsion submitted to sensitisation.

Virulent pneumococci are surrounded by capsules which are stained readily by basic dyes, such as carbol-fuchsin. During sensitisation a material is deposited around the bacteria, which is also readily stained by basic dyes, and is indistinguishable from the capsule under the microscope, though rather less regular in distribution.

When inoculated into the peritoneal cavity of the mouse, the killed raw organism scarcely excites any cell exudate; the cocci seem gradually to disappear without undergoing obvious phagocytosis. A living virulent pneumococcus culture at first excites the appearance of leucocytes but later destroys them, the struggle between the living cocci and the cells being continued during about half the time taken by the animal to succumb to a lethal dose of culture (48 hours).

Although the protective anti-bodies alone have no influence on leucocytosis, sensitisation undoubtedly renders the otherwise resistant pneumococcus

susceptible. Intraperitoneal inoculation of mice with fully sensitised pneumococcus vaccine immediately excites an outpouring of large macrophages which, in an astoundingly short space of time, within an hour at most, ingest all the sensitised cocci. The intracellular cocci gradually disappear, and in 12 to 16 hours have completely vanished, in 24 hours the character of the peritoneal exudate has reverted to normal.

The method described in a previous communication (4) of accurately titrating the protective potency of anti-pneumococcal serum was again adopted. Mice were inoculated intraperitoneally with a fixed volume (0.2 c.c.) of immunising serum, and the protection so conferred was determined by subsequent administration of measured dilutions, in broth, of a living culture of pneumococcus of standard virulence.

The minimal lethal dose for mice of 18 to 22 grams weight of the strain of Type II pneumococcus used in the experiments about to be described was one-millionth of a cubic centimetre of a blood broth culture after 16 hours' inoculation.

Mice were used as test objects. Experiments were made :—

(1) To compare the prophylactic power of a measured dose of sensitised vaccine with that of immune serum.

(2) To confirm Avery's observation (5) that all the protective substances can be absorbed from an immunising serum by the homologous type pneumococcus.

(3) To ascertain if the protective substances absorbed from a given volume of an immunising serum confer on the sensitised vaccine a protective power equal to that of the serum before exhaustion.

Immunising sera of such potency that 0.2 c.c. serve to protect mice against 200,000 minimal lethal doses of living pneumococcus culture are readily prepared by intravenous inoculation of rabbits with killed pneumococcus vaccine.

A very large number of tests have proved that 10,000 million killed pneumococci, fully sensitised by a serum of good protective and agglutinating power, will protect mice of 18 to 22 grams weight against inoculation, 16 hours later, of 0.2 c.c. of broth culture; in other words, against 200,000 minimal lethal doses

A qualitative test served to confirm Avery's observation that all the protective substances may be withdrawn from a sample of immunising serum by repeated absorption with the homologous pneumococcus, the serum losing all its protective power. Similarly it was found that the protective substances

could be removed quantitatively by absorption with vaccine, and that, on inoculation into test mice, the charge of protective substance carried by the thus sensitised vaccine was almost completely liberated. Ten thousand million pneumococcal vaccine was saturated with the anti-bodies from 0.2 c.c. of immunising serum. The sensitised vaccine successfully protected mice from 200,000 minimal lethal doses of living culture. The exhausted serum was found still to possess slight protective power, a volume representing 0.2 c.c. of the original serum, before dilution by the addition of the vaccine suspension, neutralising 1,000 minimal lethal doses. This serum was used for sensitising a further quantity of vaccine. Ten thousand million of the part-sensitised vaccine protected against no more than ten minimal lethal doses of culture. Hence 10,000 million vaccine had fixed  $1,000 - 10 = 990$  minimal protective doses of anti-body. One thousand minimal protective doses of anti-body equal in protective power 50 million sensitised pneumococcal vaccine. The fixed anti-body combined in 50 million vaccine may be calculated to be  $\frac{1}{2} \frac{1}{10} \frac{1}{10} = 4.95$  minimal protective doses. Although the figures given must be regarded only as approximate, the experiment has been repeated several times with similar results, and indicates that a small fraction of the anti-bodies in a sensitised pneumococcal vaccine—about 1/200th—remains firmly combined, the loosely associated balance being liberated on inoculation.

Garbat and Meyer (6) assert that the immunising sera produced by inoculation of rabbits, on the one hand with sensitised, on the other with raw typhoid vaccine, exhibit different properties. Experiments were made to ascertain if a similar difference in antigenic power exists in the case of raw and sensitised pneumococcal vaccines

Part of a batch of vaccine was sensitised, and increasing doses of the sensitised and unsensitised or raw vaccine were inoculated intravenously into two rabbits at 48-hour intervals; the doses consisted of 2, 4, 6, 8 and 10 thousand million organisms. At 48-hour intervals from the beginning of the experiment and prior to the inoculation of each successive dose of vaccine, the animals were bled and serum samples collected and stored on ice. The immunising power, in terms of minimal protective doses for mice, was measured by titration against known doses of living virulent pneumococci.

Chart 1 illustrates the results of these experiments. The graphic method adopted is that described previously (4), serum potency being expressed as the logarithms of the protective power in terms of minimal protective doses for mice. Vaccine doses again in millions, as in former paper.

The appearance of immunising power in the serum of the animal which

received sensitised vaccine was delayed until the eighth day after inoculation began. The level of immunity on the twelfth day was one hundred times less

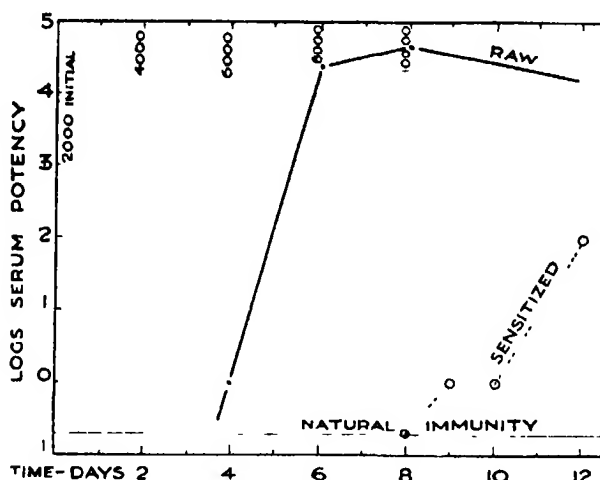


CHART 1.—Immunity in rabbit following increasing intravenous doses of raw and sensitized vaccine.

than in the case of the rabbit correspondingly inoculated with raw vaccine, nor did the serum from the sensitised vaccine rabbit, on any subsequent day, acquire so high a titre as in the animal which received raw vaccine.

Sera from rabbits inoculated with sensitised Type II pneumococcus vaccine are conspicuously deficient in agglutinin, as compared with those from animals which have received raw vaccine; none the less the former exercise full protective effect when inoculated into animals.

A very small but demonstrable quantity of complement-fixing anti-body was present in sera prepared either by inoculation of raw or sensitised vaccine, slightly but not measurably more in the serum from rabbits inoculated with raw vaccine. A series of careful tests, using serum and living culture together with guinea-pigs complement, failed to demonstrate the existence of bactericidal power in either serum.

As shown in Chart 1, serum immunity following repeated increasing doses of sensitised vaccine is delayed and diminished as compared with that following inoculation of raw vaccine.

The result in no way confirmed the favourable view taken of the action of sensitised vaccine by Besredka, and seemed to indicate that the combination of Type II pneumococcal vaccine with protective anti-bodies had led to a reduction of the antigenic power of the vaccine.

In order to investigate further the effects of sensitisation, a comparative study of the immunity following the inoculation of large single doses of sensitised and raw vaccine was undertaken.

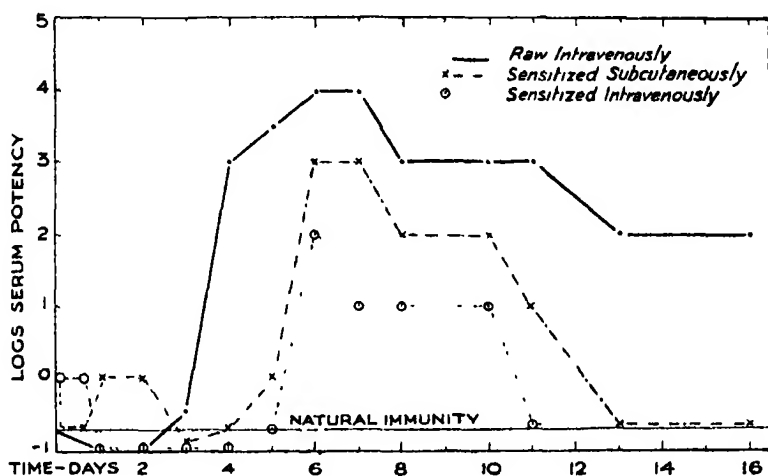


CHART 2.—Immunity following single doses of 40,000 million raw and sensitized vaccine.

Chart 2 illustrates the pooled results of two distinct experiments ; these may be summarised briefly as follows —

1. Sensitised vaccine, inoculated intravenously, exerts, in virtue of its charge of protective substances, an immediate but brief immunising effect which becomes exhausted within twenty-four hours. A phase of prolonged exhaustion of the natural non-specific anti-infectious power possessed by the serum of the normal rabbit then becomes apparent and lasts more than three days. This "neutralisation" phase is succeeded by an increase in the immunising power of the serum, which is to be attributed to the action of the pneumococcal component of the vaccine or antigen. The action culminates on the sixth day, in the course of which measurements of the protective power of serum samples prove that immunity rapidly increases.

2. Sensitised vaccine inoculated subcutaneously—the route most commonly followed in clinical practice—also sheds its protective charge and confers a brief immunity. This effect is exercised slowly and does not become fully apparent until after twenty-four hours. A period of exhaustion of natural immunity is apparent on the third and fourth day ; on the sixth day, serum samples show high protective power, surpassing that of intravenous inoculation, but not equalling the high immunity which follows inoculation of raw vaccine.

3. A prolonged period—upwards of three days—of subnormal immunity follows inoculation of raw vaccine intravenously. Protective bodies then appear suddenly in high concentration in the serum, rising to a maximum on the sixth day.

4. The level of immunity in the serum of rabbits inoculated with sensitised vaccine rapidly declines after the sixth day and is reduced to a low level by the twelfth day. On the other hand, a high level of immunity persists till the twelfth day after inoculation with raw vaccine.

It seems reasonable to conclude that, on inoculation, sensitised vaccine is separated from its charge of protective anti-bodies and afterwards functions in a manner similar to raw vaccine.

*Effect of administering immunising Serum ("passive" immunity) on the Reaction following Inoculation of Raw Vaccine ("active" immunity).*

The salient difference between the action of raw and sensitised pneumococcal vaccines as antigens appears to be that sensitisation causes a relative postponement and diminution of the antigenic power.

Experiments were made to analyse the character of the immunity following separate administration of the ready-made protective agent, *i.e.*, immunising serum, both before and after the excitation of "active" immunity by the inoculation of raw vaccine.

Chart 3 illustrates the course of events in the serum of a rabbit immunised by intravenous inoculation of 5 c.c. of immunising serum estimated to contain

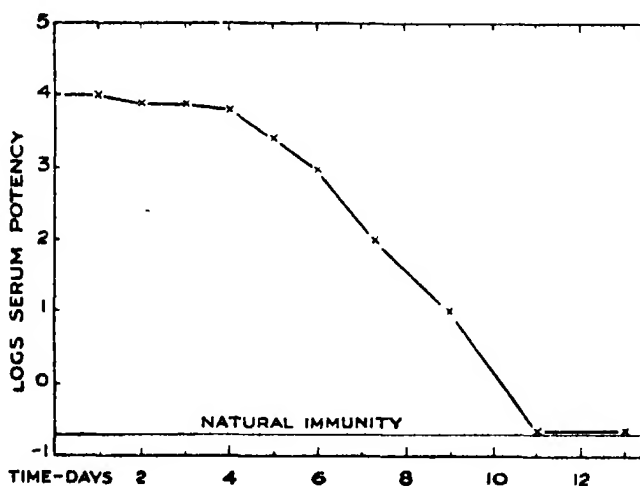


CHART 3—Immunity following 5 c.c. immunizing serum intravenously.

5,000,000 minimal protective doses of anti-body. Samples were taken at intervals of 24 hours. It will be observed that a high degree of protection was at once afforded and that the level of immunity altered but little during the first four days following inoculation, as if the immunizing substances were by some means retained in the circulating blood for a time. Protection gradually diminished, disappearing on the eleventh day.

Chart 4A illustrates the curve of immunity normally following intravenous inoculation of a single dose of 50,000 million raw pneumococcal vaccine to a rabbit. This experiment serves as a control to those recorded in which a large single dose of immunising serum (Chart 4B) was administered intravenously 24 hours after inoculation of raw vaccine and (Chart 4C) in which repeated doses of the immunising serum were given 24, 48, and 72 hours after the inoculation of raw vaccine.

In both cases a high degree of immunity followed the administration of ready-made immunising substances, the immunity persisting rather longer when the administration of immunizing serum was extended over a longer time; there was a well-marked fall in the level of serum immunity in the rabbits which had received immunising serum, in advance of the sharp rise which signalized their "active" immunity response to the large initial dose of vaccine given.

It is suggested that a simple postponement of the "active" immunity due to vaccination had resulted from the inoculation of ready-made immunising substances. In fact, the curves appear to represent a combination of the effects depicted in Chart 3 with those displayed in Chart 4A. The conspicuous dip in the curve on the fifth and sixth days (Charts 4B and 4C) represents the meeting of the descending curve expressing fall of "passive" immunity with the ascending curve of rising "active" immunity.

In order to confirm this striking result, the experiment was repeated, doses of immunising serum being inoculated on each of five successive days, beginning 24 hours after the inoculation of raw vaccine. As before, there was a conspicuous dip in the immunity curve occurring later, on the eighth day, as was to be expected from the prolonged administration of immunising serum.

Chart 5 illustrates a converse experiment to that depicted in Chart 4. An animal was protected by inoculation at intervals of 24 hours with two successive doses of serum each of a potency of one million minimal protective doses; 48 hours from the first inoculation a dose of 50,000 million raw pneumococcus vaccine was given. Rapid and complete neutralisation of immunity followed the inoculation of the raw vaccine (Chart 5B).

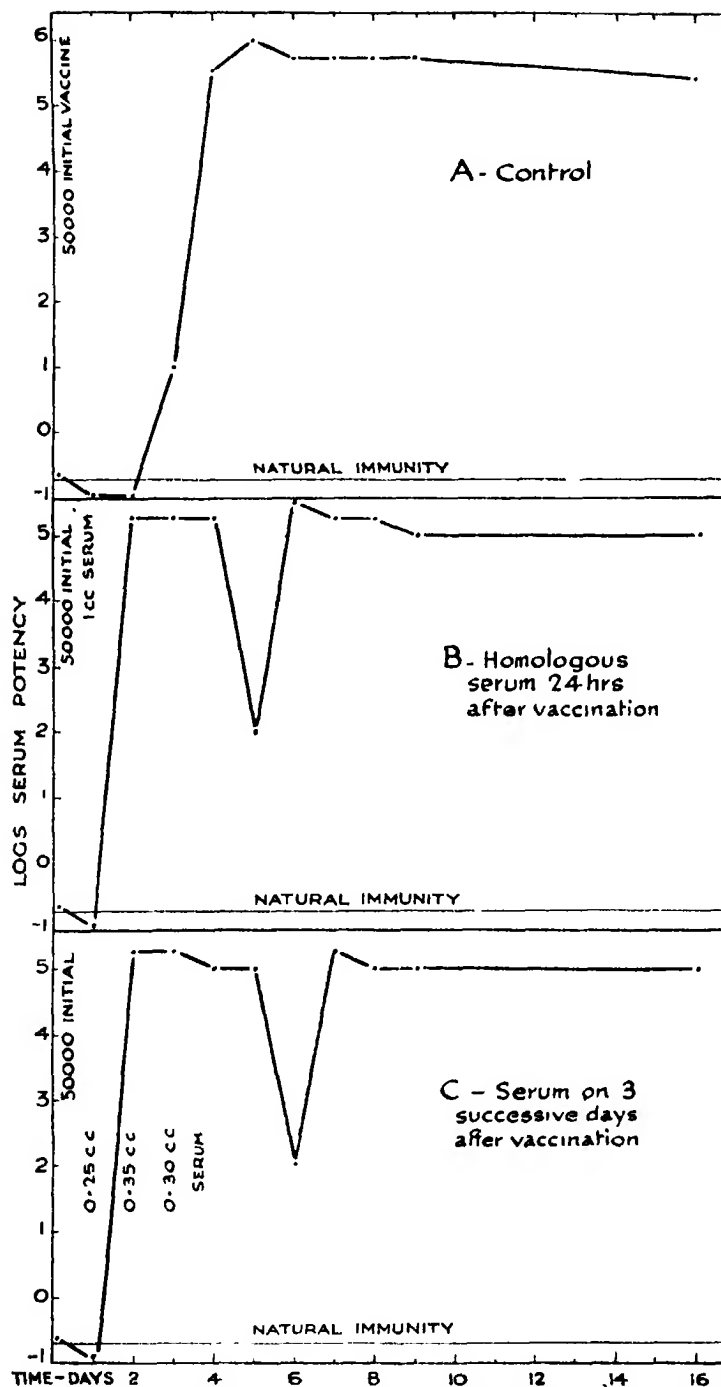


CHART 4.—Administration of immunizing serum after intravenous vaccination.



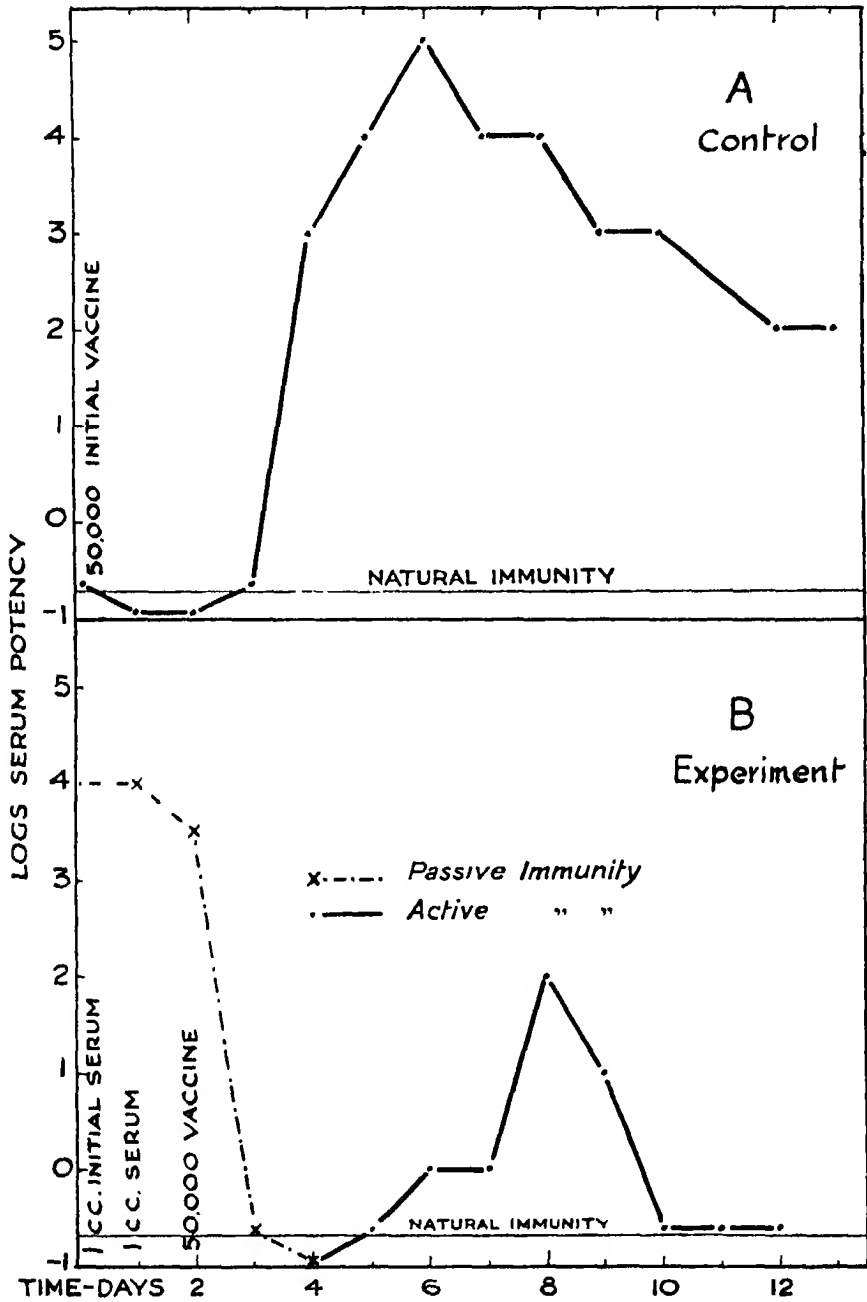


CHART 5.—Administration of homologous immunizing serum prior to vaccine inoculation.

An inductive phase of normal duration and intensity, as compared with a control animal (Chart 5A), succeeded the vaccination, but the degree of protective power, shown subsequently by serum samples, was much less than in the case of the control. The experiment indicates, therefore, that administration of ready-made protective anti-bodies to an animal, prior to vaccination, causes a diminution of the intensity of the immunity response, but no postponement such as was described in the converse experiment.

The difference in the shape of the curve of immunity in control animals, following simple inoculation of vaccine, depicted in Charts 4A and 5A, is to be ascribed to the fact that in the one case large Belgian hares were used and in the other small Dutch rabbits. The serum of these latter was severely depleted of protective anti-bodies by repeated bleedings. The batch of pneumococcal vaccine used in Experiment 5 was also known to be inferior in antigenic power to that in Experiment 4.

The diminution of the intensity of the response when immunising serum is administered prior to or simultaneously with pneumococcal vaccine, might obviously be explained as a result of destruction of a part of the sensitised vaccine or combined antigen, the remaining combined antigen separates into its two constituents, the vaccine component, as has been seen (Chart 2), then acts as an active antigen.

This simple hypothesis, however, does not serve to explain the postponement of the "actively produced" immunity, due to the stimulus of pneumococcal antigen, which follows prior or simultaneous inoculation of protective anti-bodies. Ample experimental proof has been furnished that a sensitised vaccine parts with its neutralising charge almost immediately after intravenous inoculation. Experiments previously described (4) prove that the smaller the dose of antigen, the shorter and less intense is the period of induction which precedes immunity. Were the hypothesis correct that a given quantity of sensitised pneumococcal vaccine represents a smaller effective dose of antigen than the same quantity of raw vaccine, it might be expected that the inductive period would be shorter in the former case than the latter. The reverse is the case, as shown by Chart 2, if the brief immunity conferred by the immunising substances liberated from the sensitised vaccine be disregarded.

When protective anti-bodies are administered subsequently to antigen, there is no marked reduction in the expected intensity of the immunising response due to vaccination. Presumably, that part of the pneumococcus which functions as an antigen establishes fixed contact with the cells which are responsible for the formation of protective substances and proceeds to function. The

effect of the presence of excess of protective anti-bodies, at an early stage in the immunity response, is merely to deter, that is, to slow, the rate of production of these latter without diminishing the quantity eventually produced.

The results may be summarised as follows :—

1. Administration of protective anti-bodies after the inoculation of antigen leads to postponement without conspicuous diminution of the intensity of the immunity response which normally follows administration of vaccine ("active" immunity).

2. Administration of antigen to an animal already immunised by intravenous inoculation of immune serum ("passive" immunity) leads to marked diminution but no postponement of "active" immunity.

3. If both antigen and protective anti-bodies be administered in equivalent proportions simultaneously, in the form of a sensitised vaccine, the development of "active" immunity in rabbits is both postponed and diminished as compared with control animals inoculated with an equal dose of raw vaccine.

#### *Summary.*

The pneumococcus combines proportionally with its homologous protective anti-bodies to form a "sensitised" vaccine. On inoculation, a sensitised vaccine liberates the greater part of its charge of anti-bodies, rapidly conferring a slight degree of immunity and afterwards excites an active immunity in a manner comparable with that of raw vaccine. Sensitisation, however, leads to a reduction in the intensity and to delay in the immunity response, compared with that following an equal dose of raw vaccine. These effects have been imitated by separate administration of immunising serum both before and after inoculation of vaccine.

The experiments described in this and the preceding paper have been carried out in the Research Laboratory of St. Bartholomew's Hospital by permission of Sir Frederick Andrewes, to whom and to Dr. M. H. Gordon, Consulting Bacteriologist to the Hospital, I am greatly indebted for much practical advice and help.

The work was undertaken with the assistance of the Medical Research Council, to which my grateful thanks are due.

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*The Cytology of Tar Tumours.*

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1. *Introduction.*

Numerous experiments carried out within recent years have shown that the regular application of tar to the skin of the mouse induces tumour growth, which may ultimately become malignant. The general microscopic anatomy of tar cancers, produced in the laboratories of the Imperial Cancer Research Fund, have been described by J. A. Murray and W. H. Woglom (14). These investigators have shown that the malignant condition supervenes in small isolated foci, within the area painted with tar, and is not a diffused change, affecting all the cells exposed to irritation. Further, the degree of differentiation of the epidermal cells of the tumour varies considerably. In general, the more easily the carcinomatous condition is produced, the nearer the normal is the differentiation shown by the tumour, and *vice versa*. The present research was carried out in order to compare the cytological characters of the cells of normal skin with those of tar tumours, special attention being paid to the Golgi apparatus of the cells.

## 2. *Material and Technique.*

The tar tumours of the mouse described in this paper were produced experimentally in the laboratories of the Imperial Cancer Research Fund, and the material was placed at my disposal by the Director, Dr J. A. Murray, F.R.S. The technique employed for the study of the various cell organs is summarised below.

### 1. *For Nuclear Structures*

#### *Fixation.*

Boveri's alcohol-acetic-acid fluid.

#### *Staining.*

- (a) Iron-alum hæmatoxylin.
- (b) Mann's methyl-blue-eosin.
- (c) Murray's Van Gieson Nile blue.

### 2. *For Mitochondria*

Schridde's method.

- (a) Iron-alum hæmatoxylin.
- (b) Altmann's anilin-acid-fuchsin.

### 3. *For Golgi Apparatus.\**

(a) Nassonov's method.

Unstained.

(b) Modified Kopsch method.

### 4. *For Golgi Apparatus and Nuclear Structures.*

Fixation as in 3.

Acidulated neutral red.

### 5. *For Golgi Apparatus, Mitochondria and Nuclear Structures.*

Fixation as in 3.

Altmann's anilin-acid-fuchsin and toluidin blue

## 3. *The Epidermal Cells.*

The characteristic features presented by the cell organs of the epidermal cells are associated with : --

- (a) the process of keratinisation ·
- (b) various pathological conditions of the cell, common to new growths ;
- (c) a morphological expression of interaction between tumour cell and stroma (cell polarity).

(a) The behaviour of the cell organs during keratinisation, occurring in the course of normal and malignant growth, has been described in a former paper (8). Lack of suitable material at the time prevented the study of the Golgi apparatus of the cells of tar tumours during cornification. Since, however, the

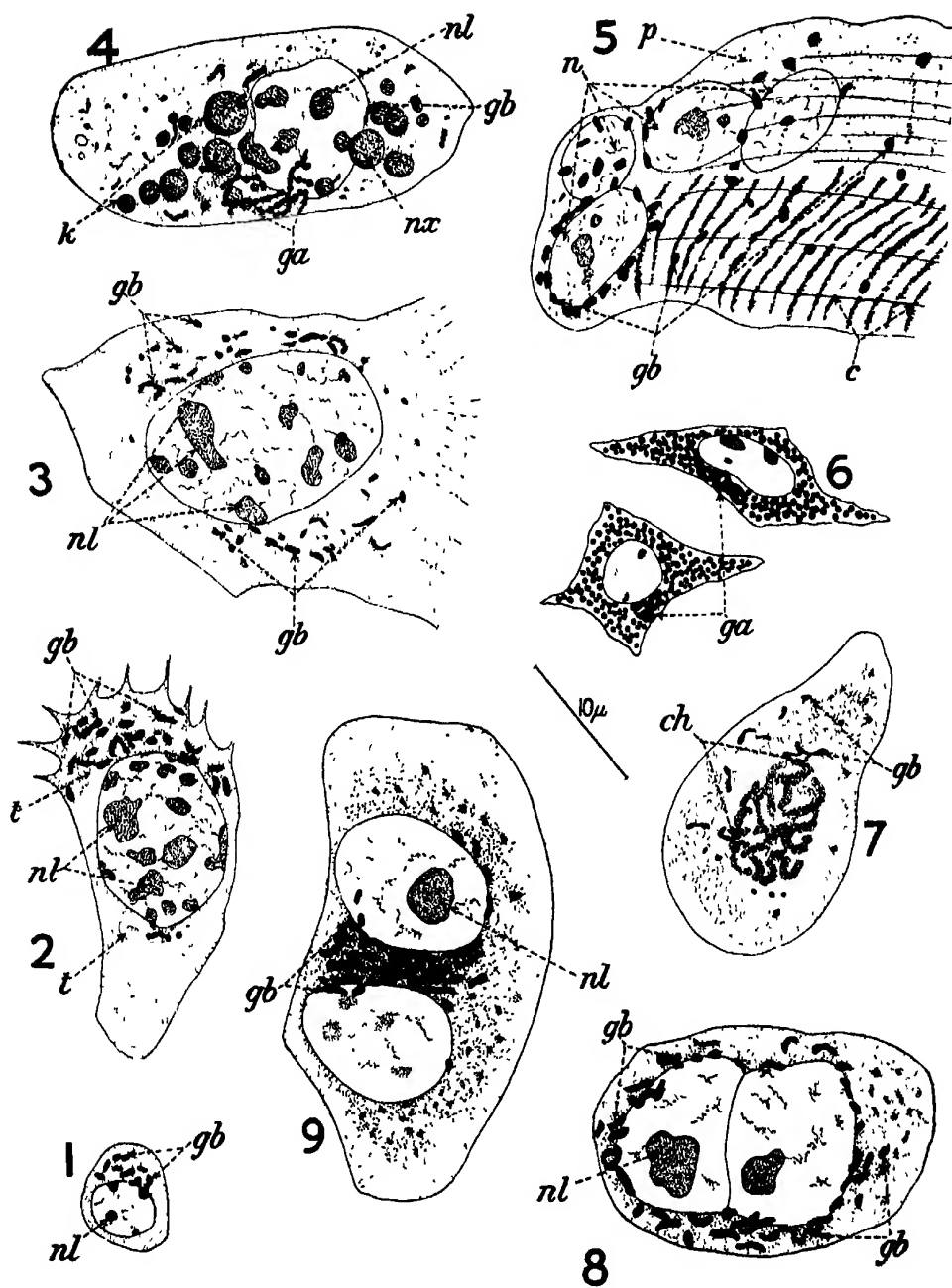
\* Full particulars of the osmic acid methods employed in this work have been given in my former papers (6, 7)

cytological processes were found to be essentially similar in the normal, hypertrophied, and malignant growths studied, there was no reason to expect any fundamental difference in the rôle of the Golgi apparatus during keratinisation in the tar tumours. Subsequent observations have shown that, as in the cases previously described, so in the tar tumours, the Golgi apparatus becomes dispersed in the form of scattered bodies at the onset of keratinisation, and that these bodies subsequently cease to be demonstrable by the osmic acid technique. This is the case in the normal skin of the mouse, in the hypertrophied zone surrounding the tar tumours, and in the tumours themselves.

The process is represented in figs. 1-4. Fig. 1 shows a normal epidermal cell of the lowest layer of the stratum mucosum. It has the Golgi apparatus in the form of scattered rodlets (*gb*) grouped towards the upper pole of the nucleus. At fig. 2 is shown a hypertrophied cell from the lowest layer of the thickened epidermis surrounding a tar tumour. Here again the Golgi bodies (*gb*), which individually are larger, are grouped at the distal end of the cell, away from the dermis. At the onset of keratinisation, with such cells approaching the surface of the skin, the Golgi bodies are scattered irregularly, as shown in fig. 3. Usually this dispersal precedes the nucleolar extrusion, which gives rise to keratohyalin, but in the cell represented in fig. 4 the apparatus is only partially disintegrated. Scattered bodies (*gb*) are seen, and also the remains of the compacted form of the apparatus (*ga*) which presents the appearance of rows of osmophil beads. The nucleolar extrusions (*nx*) giving rise to keratohyalin granules (*k*) are a conspicuous feature of this cell. Such retardation in the dispersal of the Golgi apparatus is exceptional. This particular cell was drawn from the region of the tumour, immediately adjacent to cells at the stage of keratinisation corresponding to those of the stratum lucidum of the skin.

(b) Of abnormal conditions of the epidermal cells, hypertrophy is the most pronounced. Some of the enlarged cells contain a single large nucleus (fig. 15, p. 562), others lobed nuclei (figs. 8, 14 and 17), while binucleate cells also occur (fig. 9). With the hypertrophy of the cell, the nucleus enlarges, and there is an increase in the number and size of the Golgi bodies, as can be seen from figs. 8 and 9. The mitochondria (*m*) also are more numerous in such cells, but there is little difference in their actual size (*cf.* figs. 15 and 16). The nucleolar content of the nuclei of hypertrophied cells is often considerably increased, as can be seen from the two cells shown in fig. 15.

In degenerating cells, which occur so commonly in some growths, the pathological conditions of the nucleus are associated with corresponding changes in



## Figs. 1-9.—Various Forms of the Golgi Apparatus in the Different Cells of Tar Tumours.

Figs. 5, 6 and 9 were drawn from preparations fixed and osmicated according to Nasonov's method, the remainder from modified Kopsch preparations, stained with acidulated neutral red.

- Fig. 1.—Cell from the lowest layer of the stratum mucosum of the skin, showing the grouping of the Golgi bodies (*gb*) at the upper pole of the nucleus.
- Fig. 2.—Hypertrophied cell from a similar position in the thickened epidermis surrounding a tar tumour; grouping of Golgi bodies (*gb*) the same as in fig. 1. (1960, I.C.R.F.)
- Fig. 3.—Cell at the commencement of keratinisation, from the thickened epidermis surrounding a tar tumour. Golgi bodies (*gb*) spread throughout the cytoplasm. (1968, I.C.R.F.)
- Fig. 4.—Epidermal cell from a zone of keratinisation within a tar tumour, showing retardation in dispersal of the Golgi bodies (*gb*), and the formation of keratohyalin (*k*) from nucleolar extrusions (*nz*). (1968, I.C.R.F.)
- Fig. 5.—Portion of striated muscle fibre from a region of infiltration by tumour cells, showing the increase of sarcoplasm (*p*) through which the Golgi bodies (*gb*) have become dispersed (1969, I.C.R.F.)
- Fig. 6.—Two mast cells from a tar tumour, showing the compacted form of the Golgi apparatus (*ga*). (1969, I.C.R.F.)
- Fig. 7.—Prophase of mitosis in a tumour cell, showing the Golgi bodies (*gb*) scattered through the cytoplasm. (1968, I.C.R.F.)
- Fig. 8.—Hypertrophied tumour cell with bilobed nucleus, and Golgi bodies (*gb*) scattered around the nuclear membrane. (1969, I.C.R.F.)
- Fig. 9.—Hypertrophied binucleate cell with Golgi bodies (*gb*) grouped between the two nuclei. (1969, I.C.R.F.)

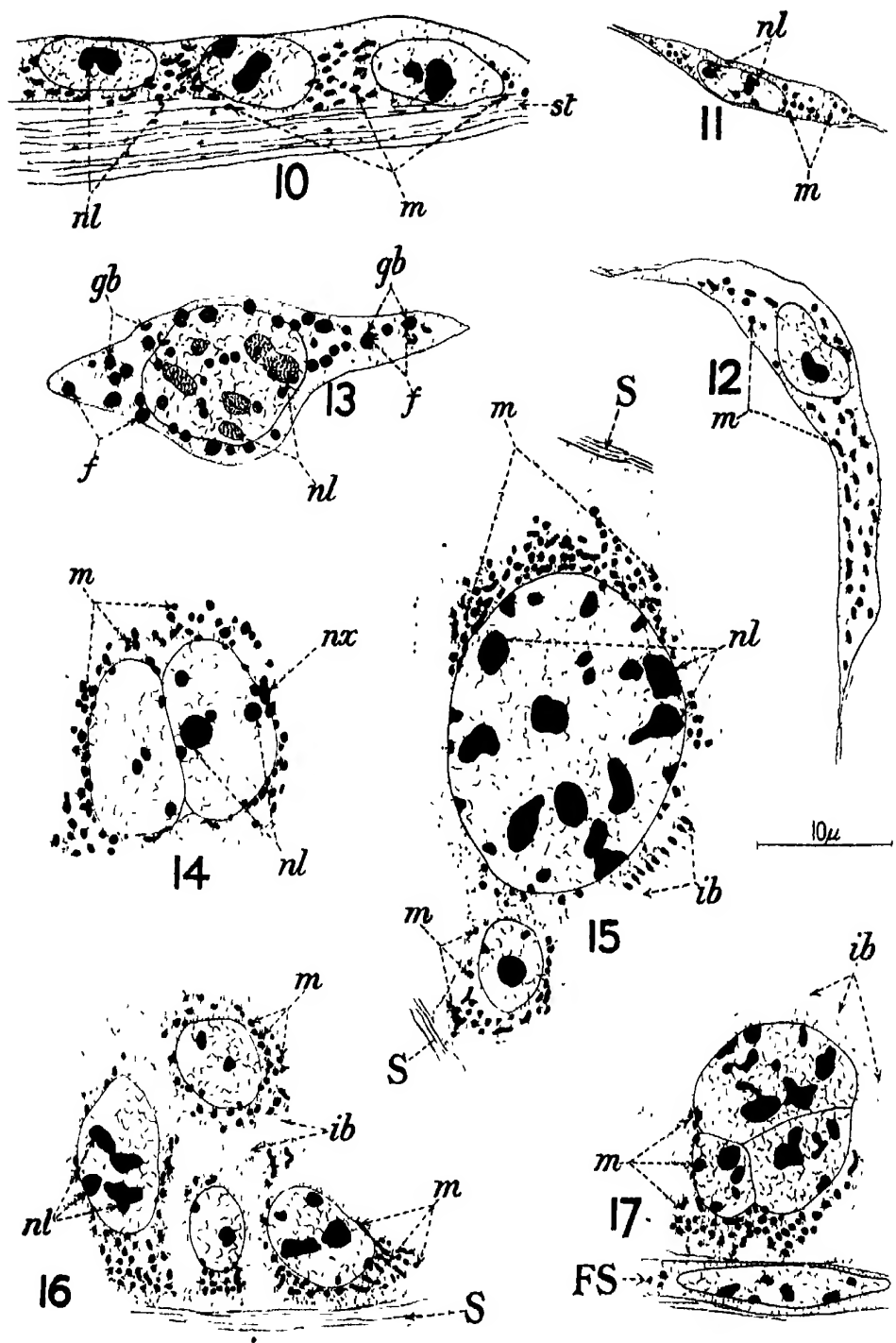
All figures were drawn by the aid of a camera lucida. A length of  $10\mu$ , enlarged to the same extent as the figures, is included, as an indication of the magnification employed.

Both capitals and small letters have been employed; the former refer to cells and tissues, the latter to cell organs. C, connective tissue; E, epidermal cell; F, fibroblast; L, lymphocyte; M, striated muscle fibre; N, endothelial cell; P, polymorphonuclear leucocyte; R, erythrocyte; S, stroma of tumour.

c, cross striation of muscle fibre; cf., collagen fibril; ch., chromosomes; f, droplet of fat; g, droplet of fatty secretion covered with the lipid substance of the Golgi apparatus; ga, Golgi apparatus; gb, Golgi body; gg, granule resulting from degeneration of a Golgi body; k, keratohyalin granule; m, mitochondria; n, nucleus; nl, nucleolus; nz, nucleolar extrusion; tb, intercellular bridge of cytoplasm, of epidermal cell; o, osmophil zone of cytoplasm surrounded by Golgi bodies; p, sarcoplasm, s, fatty secretion; st, sarcostyle of muscle fibre; t, trophospongium; v, vacuole representing the initial phase in the formation of a secretion droplet.

the cytoplasmic organs. Generally at the commencement of retrogressive processes the mitochondria present a vesicular appearance, and later disappear altogether, while the Golgi apparatus breaks up into granules, which persist until the cell becomes definitely necrotic. Such changes are found in rapidly growing tumours, where the growth and invasion of the epidermal cells precedes the formation of a vascularised stroma, with the result that the cells furthest





**Figs. 10-17.—Cytoplasmic Inclusions (principally Mitochondria) in the Various Cells of Tar Tumours.**

With the exception of fig. 13, which was drawn from a modified Kopsch preparation, the remainder of the figures of this plate were drawn from Schridde preparations, stained with iron hæmatoxylin

Fig. 10.—Part of a regenerating striated muscle fibre, showing the proliferation of mitochondria (*m*) accompanying the formation of sarcoplasm.

Fig. 11.—Normal fibroblast, showing mitochondria (*m*).

Fig. 12.—Hypertrophied fibroblast, from the stroma of a tar tumour, with an increased number of mitochondria (*m*) (16710, I.C.R.F.)

Fig. 13.—Epidermal cell from a tar tumour, showing the formation of a fatty secretion (*f*) under the influence of the Golgi bodies (*gb*).

Fig. 14.—Epidermal cell from a tar tumour. Mitochondria (*m*) scattered irregularly. (16710, I.C.R.F.)

Fig. 15.—Normal and hypertrophied cells from a tar tumour. The mitochondria of each cell are collected towards that part of the cytoplasm next the stroma (*S*). (16710, I.C.R.F.)

Fig. 16.—Group of epidermal cells bordering on the stroma (*S*), showing the polarised distribution of the mitochondria (*m*) in the cells next the stroma. (16710, I.C.R.F.)

Fig. 17.—Epidermal cell with multilobed nucleus. The mitochondria are accumulated in that part of the cytoplasm bordering on a fibroblast of the stroma (*FS*). (16710, I.C.R.F.)

removed from the blood stream degenerate and die. This is happening to the epidermal cells on the right of fig. 34 (p. 574). The change which takes place in the Golgi apparatus under these conditions is essentially similar to that described by Da Fano as occurring in re-absorbing tumours (1).

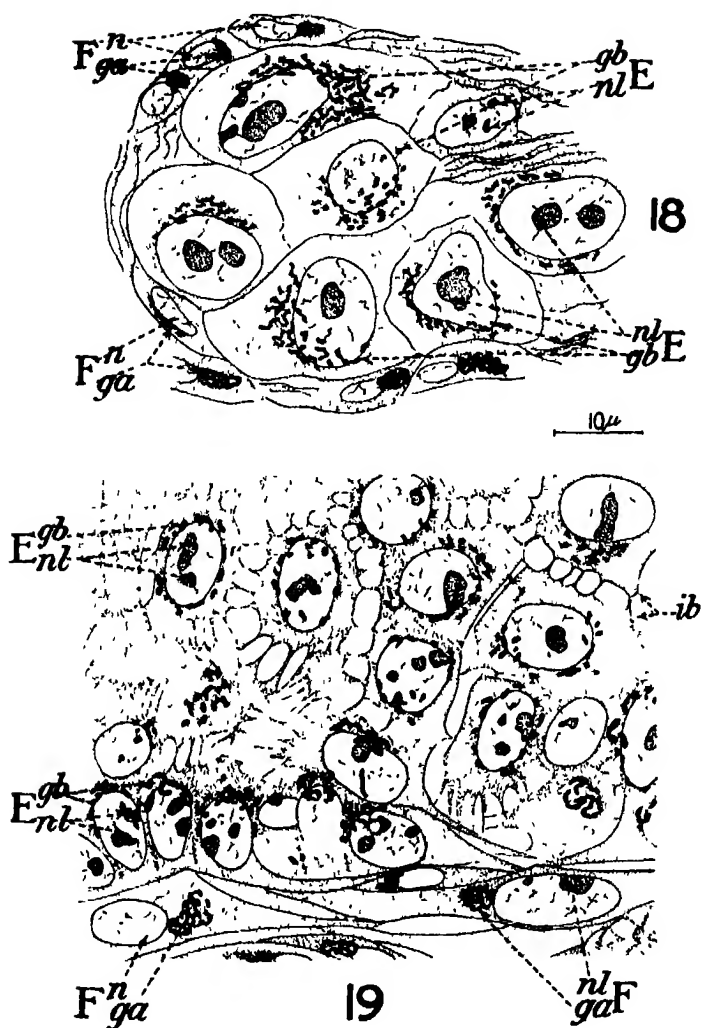
(c) There is considerable variation in the manner in which the cytoplasmic organs are disposed in the epidermal cells. In cells of the lowest layer of the rete mucosum of the epidermis, as was pointed out in my former paper, the Golgi apparatus occurs either as a juxta-nuclear group of rodlets, or as a network, at the distal end of the cell, while the mitochondria are heaped up at the opposite end, next the connective tissue of the dermis. A similar polarised distribution of the cytoplasmic organs exists in tumour cells in contact with the stroma. The mitochondria are collected at the end of the cell bordering on the stroma, and the Golgi apparatus is disposed at the opposite end. This appears to be the case in malignant, as well as non-malignant tumours. At fig. 19 (p. 565) are shown the lowest layers of epidermal cells of a tar tumour. The Golgi bodies (*gb*) of the cells (*E*), next the fibroblast (*F*) of the dermis, are seen to be collected at the upper pole of the nucleus, while in the more superficial cells the Golgi bodies are irregularly scattered around the nucleus, often in contact with the nuclear membrane. Fig. 16 shows the mitochondria in cells from a similar area of the tumour. The accumulation of the mitochondria (*m*), towards

that end of the cell adjacent to the stroma (S), is a conspicuous feature. The cell furthest from the stroma, in this figure, is seen to have mitochondria fairly regularly dispersed around its nucleus. A similar polarised distribution of mitochondria has been observed in hypertrophied cells, in areas of tumours where the stroma is well developed. Examples are shown in figs. 15 and 17. A much enlarged cell, together with a normal-sized epidermal cell, is seen in fig. 15. The mitochondria (*m*) of both these cells are collected at opposite ends of the cells, next the stroma (S). A similar polarity in the distribution of mitochondria is shown in fig. 17, where is represented a cell with a lobed nucleus. This cell is in contact with a fibroblast of the stroma (FS).

In contrast to this arrangement of the mitochondria is the scattered distribution of these cell organs shown in fig. 14. This cell, also, contains a lobed nucleus. It, however, occupies a position removed from the stroma: hence the absence of polarity.

In general, therefore, it can be concluded that where epidermal cells are in contact with the dermis, or a well developed stroma, there is established a polarised distribution of the cytoplasmic organs. When the epidermal cells are remote from either stroma or dermis, usually both mitochondria and Golgi bodies are scattered irregularly. The Golgi apparatus, however, presents a greater variation in its form and manner of distribution than the mitochondria. In slow growing tumours the majority of the cells have the elements of the Golgi apparatus irregularly scattered throughout their cytoplasm. Fewer cells present the appearance of the Golgi apparatus as an excentric juxta-nuclear group of rodlets. Instances of the two types occurring in hypertrophied cells are shown in figs. 8 and 9 (p. 560). The cell represented in the former has a bilobed nucleus surrounded by the Golgi bodies (*gb*), the latter shows a binucleate cell with the Golgi bodies collected together, between the two nuclei. During mitosis, no attraction between the Golgi bodies and the centrosomes has been observable. Fig. 7 shows a prophase of mitosis, the apparatus being represented by scattered rodlets or granules (*gb*). Such is the form it presents throughout mitosis.

A relatively far greater number of cells of the more malignant tumours especially in areas where active infiltration of the underlying tissues is proceeding, have the apparatus in the form of a compacted group of rodlets. A group of such cells surrounded by their stroma is represented in fig. 18. This same form of the Golgi apparatus is seen in some of the epidermal cells in fig. 34 (p. 574), which are represented penetrating the muscle fibres (M) of the panniculus carnosus. Where the apparatus presents this form, the region



Figs. 18 and 19.—Variations in the Form of the Golgi Apparatus in Epidermal Cells from Different areas of Tar Tumours.

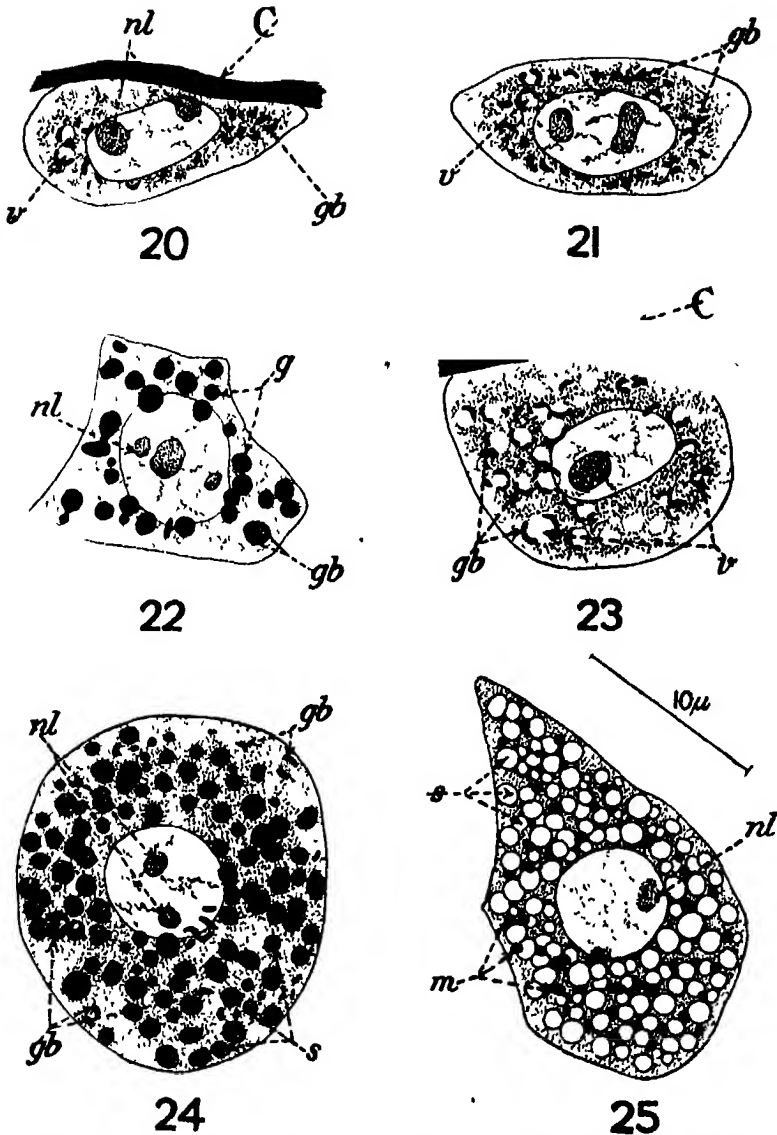
Fig. 18.—Group of epidermal cells (E) infiltrating tissues underlying the tumour. Golgi apparatus in the form of a compact group of rodlets (gb). (1969, I.C.R.F.)

(Preparation: fixation and osmication by Nassonov's method, stained with neutral red.)

Fig. 19.—Lowest layers of epidermal cells of a tar tumour. (1968, I.C.R.F.)

The cells of the dermis, next the fibroblasts, have their Golgi bodies (gb) collected at the upper poles of the nuclei. The other epidermal cells have the Golgi bodies (gb) scattered around their nuclei.

(Preparation: modified Kopsch, stained with acidulated neutral red.)



Figs. 20-25.—*The Secretion Process in the Sebaceous Gland Cells of the Normal and Hypertrophied Skin.*

Figs. 20-24 were drawn from modified Kopsch preparations. Fig. 23 is from a section bleached with hydrogen peroxide, and stained with neutral red. The other four figures were drawn from sections mounted unstained. Fig. 25 represents a cell from a Schridde preparation, stained with iron hæmatoxylin.

Fig. 20.—Cell at the beginning of the secretion process. The Golgi bodies (*gb*) are beginning to scatter, and some of them appear applied to the surface of clear vacuoles (*v*).

Fig. 21.—Cell with the Golgi bodies (*gb*) more dispersed and increased in numbers.

Fig. 22.—Cell with the fatty secretion granules (*g*) apparently covered over by the osmophil substance of the Golgi apparatus

Fig. 23.—A later stage in secretion, showing the relation of the Golgi bodies (*gb*) to the formation of the secretion (*v*).

Fig. 24.—Cell filled with fatty secretion droplets (*s*). Some Golgi bodies (*gb*) applied to the surface of the secretion granules (*s*), others free in the cytoplasm.

Fig. 25.—Cell filled with fatty secretion (*s*), the blackening due to the osmic acid having been removed with turpentine. Mitochondria (*m*) scattered amongst the secretion (*s*)

of the cytoplasm bounded by its constituent rodlets (*o*) often appears faintly osmophil.

#### 4. *The Sebaceous Gland Cells.*

Hypertrophy of the sebaceous glands is a marked feature of the thickened epidermis surrounding tar tumours. The secretory process in such glands is essentially the same as in the normal glands. The fatty secretion appears in the form of granules, in intimate relationship with the Golgi apparatus, in an almost identical manner to the yolk of certain molluscan oöcytes, as described by Gatenby (3, 4) and myself (9).

At the onset of secretory activity, the Golgi apparatus, which is in the form of curved osmophil bodies (*gb*), spreads throughout the cytoplasm, as shown in fig. 20. Apparently there takes place a proliferation of the Golgi bodies giving rise to the appearance seen in fig. 21. At these early stages certain of the Golgi bodies (*gb*) seem to be applied to the surface of vacuoles (*v*). Such vacuoles are believed to be the first indication of the secretion, forming in relationship with, or under the influence of, the Golgi apparatus. As secretion proceeds the Golgi bodies continue to increase in number, and there are formed fresh vacuoles, surrounded by the curved elements of the apparatus, as represented in fig. 23). The material of the vacuoles undergoes a chemical change resulting in the formation of fat, so that the vacuoles in the osmic impregnated material exhibit a progressive blackening as secretion proceeds (fig. 24). This blackening can be removed by treating sections with turpentine. Such cells then appear as in fig. 25. Short treatment with turpentine, however, fails to remove the blackening due to the impregnation of the Golgi apparatus. Fig. 25 does not show the apparatus, as it was drawn from a Schridde preparation, but a similar cell from a preparation with the apparatus impregnated would show a certain number of curved osmophil bodies, applied to the periphery of many of the vacuoles.

From the appearance of certain cells, such as that shown in fig. 22, it seems that the fatty secretion of the sebaceous gland cell forms inside a hollow spherical shell formed of the lipid material of the Golgi apparatus (*g*). Probably the

structure shown in fig. 23, of vacuoles, partly surrounded by osmophil material, is not a true representation of the condition in the living cell. It seems possible that the secretion forms inside a fine lipid shell, which functions as a semi-permeable membrane, and that this lipid membrane becomes somewhat distorted during fixation and prolonged treatment with osmic acid. The lipid shell shrinks, becoming thicker in some parts, entirely absent in others, and assumes, in sections, the form of curved rodlets, closely applied to the circumference of the secretion granules. Fig. 24 shows a cell with a considerable number of fatty secretion granules (*s*), many of which have the so-called Golgi bodies (*gb*) applied to their surfaces. Other elements of the apparatus are seen scattered in the cytoplasm (*gb*), while in a few places Golgi bodies are seen partly surrounding an osmophil zone, which indicates an early stage in secretion formation.

Preparations have been studied with the view to determining the part played in the secretion process by the other cell organs, the nucleus and mitochondria. Previous workers have described the mitochondria as giving rise to fat globules. J. A. Murray described this process in a transplantable sarcoma of *Cavia* (12), and Schreiner practically the same thing in the fat cells of *Myxine* (16). Then Hirschler (5) has described the formation of yolk in Ascidian oöcytes as resulting from the fusion of Golgi bodies with swollen mitochondria. It seemed at first feasible that something of this kind might occur in the sebaceous gland cells, and both mitochondrial and Golgi apparatus preparations were studied for indications of interaction on the part of the cytoplasmic organs. Nothing, however, has been observed in support of this assumption. At the beginning of secretion the mitochondria increase in numbers, and later on there are relatively fewer. A cell with the mitochondria (*m*) stained is shown in fig. 25. As more secretion is formed they become even fewer still, but actual transition stages between them and fat globules are absent from my preparations.

The nucleus exhibits the usual changes observable during secretion. There is a progressive diminution of its chromatin content, and nucleolar extrusion takes place.

Besides the secretion which goes on within the normal and hypertrophied sebaceous glands, a similar process is observable in groups of cells within the tumours. Such cells are often considerably enlarged. In some cases they possibly represent remains of the sebaceous glands, but it seems that fat may be formed in degenerating epidermal cells, in exactly the same manner as occurs normally in the sebaceous glands.

An epidermal cell from a tar tumour exhibiting this process is shown in fig. 13 (p. 562). The morphological aspect of the secretion process, it will be observed, is essentially the same as shown in figs. 22 and 24, which were drawn from sebaceous gland cells.

### *5 Cells of the Stroma of Tar Tumours.*

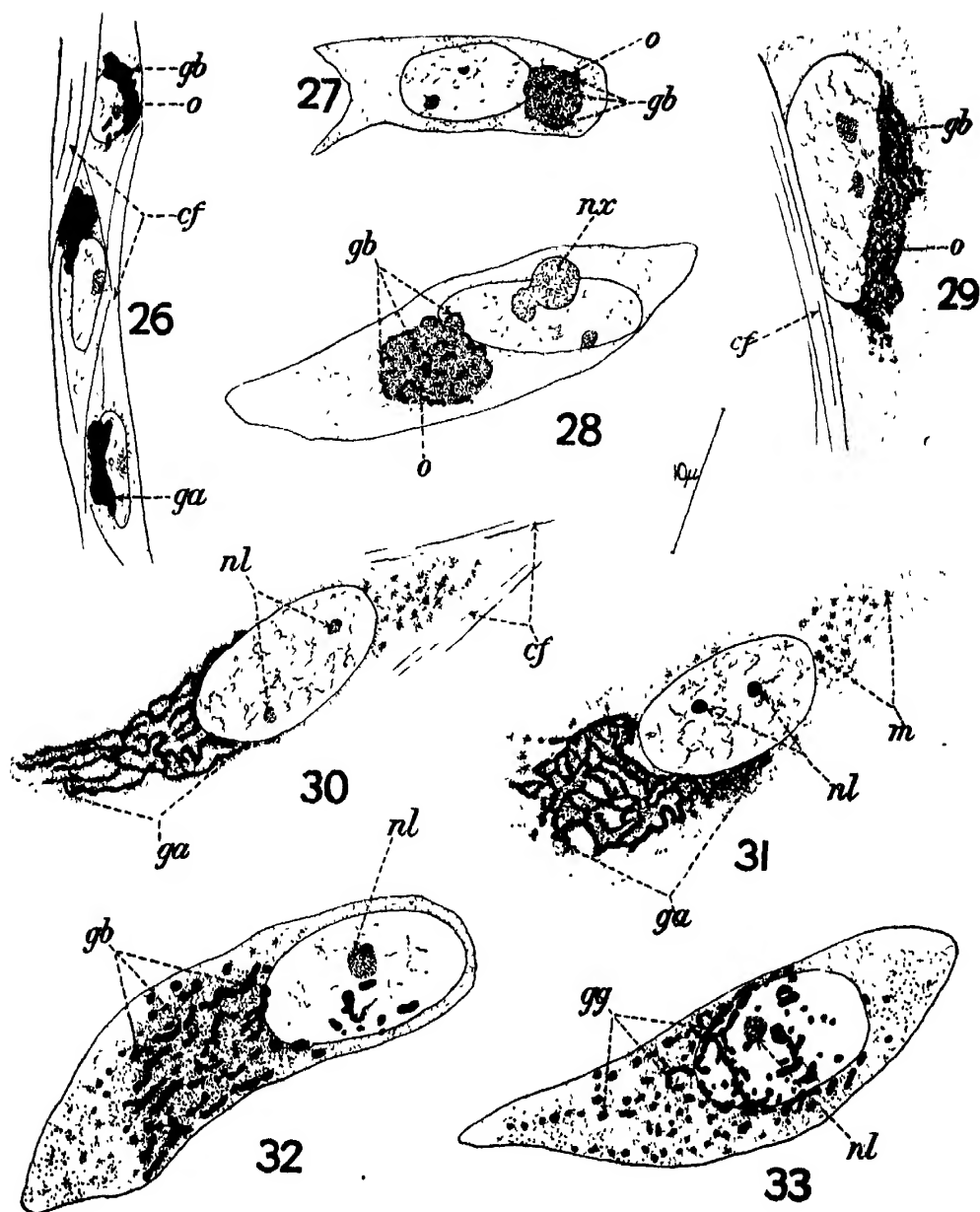
The cells showing the greatest deviations from the normal in the stroma of tar tumours are the fibroblasts and endothelial cells. Just as different tumours vary in the extent of the hypertrophy of the epidermal cells, so there is a corresponding variation as far as the stroma is concerned. In general, the more malignant the tumour the greater is the extent of the hypertrophy of its cells, especially of the fibroblasts and endothelial cells of the stroma.

*The Fibroblasts.*—In preparations made for the demonstration of the Golgi apparatus it is extremely difficult, in certain cases, to distinguish between enlarged fibroblasts and endothelial cells, as they present a very similar appearance. An attempt has been made, nevertheless, to indicate in figs. 26–33 what is regarded as the sequence of changes undergone by fibroblasts in the course of hypertrophy terminating in degeneration and necrosis. Fig 26 shows the normal fibroblasts surrounded by collagen fibres (*cf*). Each cell has a Golgi apparatus (*ga*) in the form of rodlets, closely applied to an osmophil area of cytoplasm (*o*). When deeply impregnated with osmium dioxide the apparatus appears dense black, as shown in the lowest of the three cells in fig. 26. As the cells enlarge, the constituent elements of the apparatus (*gb*), as well as the osmophil area of cytoplasm (*o*) which they enclose, are more easily distinguishable (figs. 27 and 28). During these initial phases of growth nucleolar extrusion occurs as shown at “*na*,” fig. 28.

The next stage of growth is characterised by a proliferation of the elements of the Golgi apparatus, which is represented in fig. 28. This is believed to be followed by a process of anastomosis, in which the Golgi bodies run together, as is seen occurring in fig. 29, and give rise to a complicated reticulate structure (*ga*) such as that shown in figs. 30 and 31. The fate of the osmophil cytoplasm is difficult to follow. It may be that it persists as a fine layer surrounding the reticulum of the apparatus, or else it may form the basis of the reticulum over which the material of the apparatus is spread in a similar manner to the insulating material covering an electric wire.

The maximum growth attained by fibroblasts rarely exceeds that represented





**Fig. 26-33.**—Cells believed to be Fibroblasts, showing what is regarded as the Sequence of Changes undergone during Hypertrophy, and Subsequent Degeneration.

All figures drawn from preparations made by the method of Nasonov

**Fig. 26.**—Normal fibroblasts surrounded by collagen fibres (*cf*).

**Figs. 27 and 28.**—Enlarged fibroblasts, showing the greater number of Golgi bodies (*gb*), and the increased size of the osmophil zone of cytoplasm (*o*) enclosed by them.

**Fig. 29.**—Fibroblast, showing the Golgi apparatus in a form regarded as transitional between that of figs. 28 and 30.

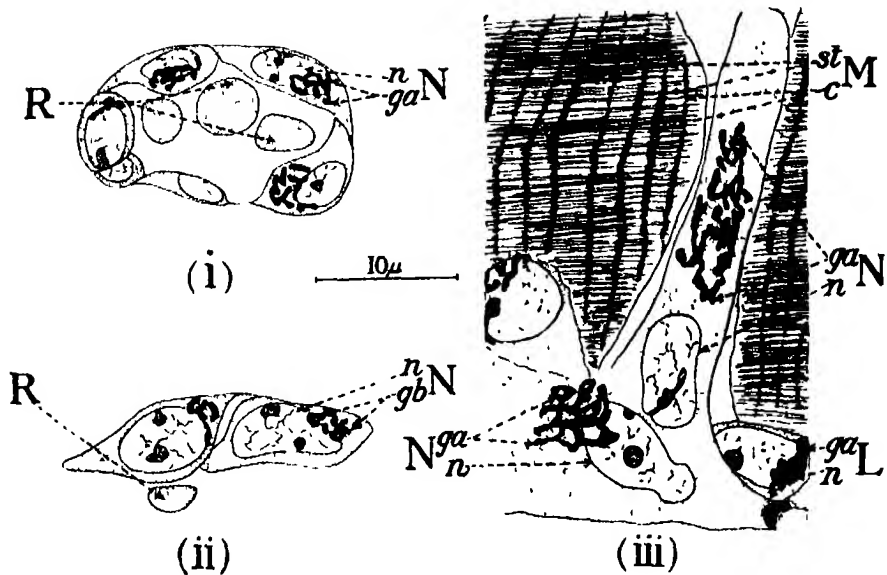
**Figs. 30 and 31.**—Hypertrophied fibroblasts showing the Golgi apparatus (*ga*) in the form of a network.

**Figs. 32 and 33.**—Consecutive stages in the degeneration of hypertrophied fibroblasts. The reticulate apparatus has broken up to form rodlets (*gb*), which are fragmenting, and giving rise to osmophil granules (*gg*).

by the cell shown in fig. 31. Many of the cells about this stage show indications of degeneration. In them the Golgi apparatus breaks up into rodlets and granules (*gb*) as seen in fig. 32, and as the retrogressive process proceeds, the osmophil bodies become more markedly granular (fig. 33), while the nucleus exhibits the usual features of degeneration.

Changes in the mitochondrial content of fibroblasts accompany alterations in the Golgi apparatus. With growth of the cytoplasm the mitochondria increase in numbers, and become slightly larger. This is evident from figs. 11 and 12 (p. 562). The former figure represents a normal fibroblast, and the latter a hypertrophied one. In both cells the mitochondria (*m*) are scattered throughout the cytoplasm, but in the larger cell there are more of them, and individually they are slightly larger and tend to assume the filamentous form.

**Endothelial Cells.**—Hypertrophy of the endothelial cells is of common occurrence, especially in the more malignant tumours. Endothelial cells (*N*) little larger than the normal are shown in the accompanying figure at (i) and (ii): the former represents a transverse section of a capillary, and the latter two cells drawn from a capillary cut longitudinally. The Golgi bodies (*gb*), it will be observed, are grouped together as in the fibroblasts, and there is a certain amount of anastomosis of the rodlets. Very much enlarged endothelial cells (*N*) are shown at (iii) same figure. This figure was drawn from a section of a tumour which was invading the panniculus carnosus. The two large endothelial cells are seen crossing the striated muscle fibre (*M*). The Golgi apparatus (*ga*) of the endothelial cells is much enlarged, and forms a reticulate structure resembling that of the enlarged fibroblasts.



The Golgi Apparatus in Endothelial Cells.---(i) 'Transverse section of a blood capillary (N—endothelial cell, R—erythrocyte); (ii) two endothelial cells (N), from a capillary cut longitudinally; (iii) endothelial cells (N) crossing striated muscle fibres (M), in the region of infiltration by tumour cells (L—lymphocyte)

ga—Golgi apparatus; gb—Golgi body; n—nucleus.

## 6 Reaction of Muscular Tissue to Infiltration by Tumour Cells.

Penetration of the panniculus carnosus by the tumour cells produces a marked reaction in the muscle fibres. These changes resemble those described as occurring in tissue cultures of muscle. Fig. 34 shows tumour cells (E) pushing their way in between the muscle fibres (M), causing disorganisation of the muscular tissue. This is followed by growth of the sarcoplasm at certain parts of the muscle fibre, which is associated with an increase in the number of the nuclei, probably due to amitotic nuclear division taking place. With growth of the sarcoplasm there is an increase in the number of mitochondria, and also in the Golgi bodies. Fig. 5 (p. 560) shows the terminal portion of a damaged muscle fibre. The Golgi bodies (gb) are spreading out from around the nuclei (n), into the sarcoplasm (p). The same process is seen taking place in the muscle of figs. 35 and 36. The early phase of dispersal of the Golgi bodies (gb) is shown at M in fig. 35, while later stages are shown at M in fig. 36, where the Golgi bodies are mostly scattered throughout the sarcoplasm away from the nuclei.

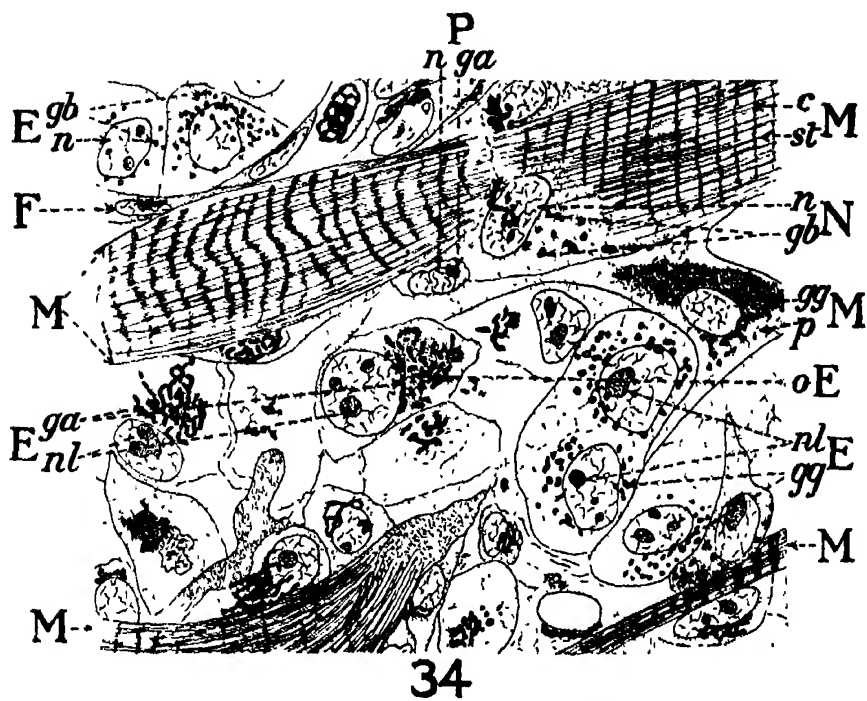
An early stage in the proliferation of mitochondria is represented at fig. 10 (p. 562). Most of the mitochondria (*m*) are filamentous in form at this stage.

With the disorganisation of the muscle tissue there is a gradual disappearance of the cross striations (*c*), and the sarcostyles (*st*) become much more conspicuous than normally. The nuclei and surrounding sarcoplasm ultimately degenerate. The sarcoplasm of such cells becomes blackened by osmic acid, and deeply impregnated granules (*gg*) apparently represent the remains of the Golgi apparatus.

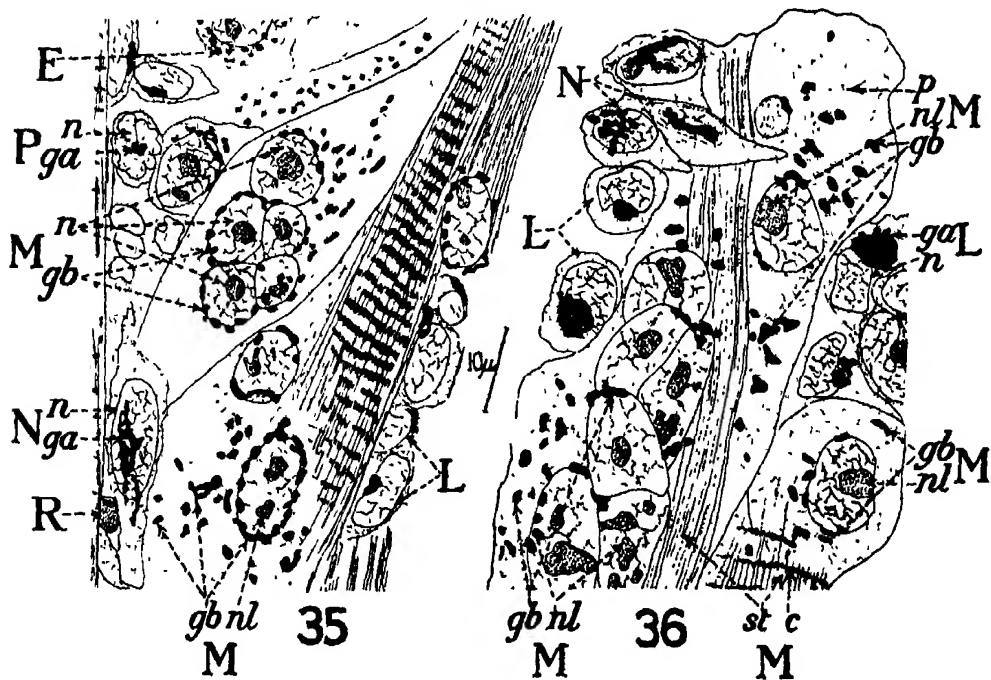
The degenerating muscle becomes infiltrated by polymorphs (*P*) and cells of the lymphocyte series (*L*) (figs 34 to 36).

#### *7. Cell Hypertrophy and Malignancy.*

Murray and Woglom have pointed out that the degree of differentiation of the epidermal cells of tar tumours varies considerably. In general, the more easily the carcinomatous condition is induced, the more dedifferentiated are the cells (14). The extent to which hypertrophy and other pathological conditions of the cell occur also vary in different growths. Roussy and his collaborators have called attention to differences in the stroma of benign and malignant tumours (15). In the more rapidly growing neoplasms the stroma is less perfectly differentiated, more cellular in character, and the fibroblasts and endothelial cells especially are often hypertrophied. The form of many of the cells calls to mind the features presented by cells in tissue cultures. Drew has shown that the addition of autolysed tissue extract to a culture accelerates growth to a marked degree (2). Probably some such medium is elaborated by the cells of rapidly growing carcinomata, as has been suggested by J. A. Murray (13). Possibly also cells undergoing necrosis release substances with similar properties. Many such cells occur in malignant growths, owing largely to their growing more rapidly than the capillaries which supply nourishment. Death and autolysis of some of the cells may, therefore, stimulate the growth of surviving cells and accelerate the formation of the stroma.



34



35

36

**Figs. 34-36.**—*Penetration of Striated Muscle Tissue by Tumour Cells.*

All figures drawn from Nasonov preparations stained with neutral red.

**Fig. 34.**—Tumour cells (E) penetrating the muscular tissue shown at M, at the top and bottom of the figure.

**Figs. 35 and 36**—Reaction on the part of the muscle tissue (M) to invasion by the tumour. The figures show the increase in the number of muscle cell nuclei (Mn), and of the Golgi bodies (M, gb), which spread from around the nuclei into the newly-formed sarcoplasm (Mp).

8. *Summary.*

The cellular anatomy of tumours produced by painting regularly the skin of mice with tar, has been studied. The observations that have been made on the cells of these neoplasms are as follows.—

1. *Epidermal Cells*—The characteristic features of their cell organs are associated with :

- (a) *Keratinisation*—During this process the Golgi bodies (gb), if originally grouped together (figs. 1 and 2), become scattered throughout the cytoplasm (fig. 3), and finally cease to be demonstrable by the osmic acid technique. When the dispersal of the apparatus is retarded, it may be recognisable during the formation of keratohyalin (k) from nucleolar extrusions (nz) (fig. 4). The behaviour of the cell organs during keratinisation is essentially the same in the normal skin, in the hypertrophied epidermis surrounding tar tumours, and in the tumour itself.
- (b) *Pathological States of the Cell.*—Cell hypertrophy is of common occurrence. Such cells contain larger and more numerous Golgi bodies (gb) (figs. 8 and 9), and mitochondria (m) are more numerous, but show little difference in size (figs. 14-17). During degeneration the mitochondria become vesicular, and then fail to stain: the Golgi bodies break up into granules (gg), which persist in the cell usually until necrosis (fig. 34).
- (c) *A Morphological expression of interaction between tumour cell and stroma* (cell polarity).—Where epidermal cells are in contact with the dermis, or a well-developed stroma (S), there is established a polarised distribution of the cytoplasmic organs. Compare Golgi apparatus (ga) in figs. 18 and 19: and mitochondria (m) in fig. 14 with those of figs. 15-17.

2. *The Sebaceous-Gland Cells.*—Hypertrophy of the sebaceous glands is a marked feature of the thickened epidermis surrounding tar tumours. The

secretory process is essentially the same as in the normal glands. The Golgi bodies (*gb*) scatter throughout the cytoplasm at the onset of secretion, and increase in numbers. The fatty secretion (*s*) appears in intimate relation with these Golgi bodies. Mitochondria do not appear to be directly concerned in the formation of the secretion (figs. 20-25). The formation of droplets of a fatty substance (*f*) occurs in the same fashion in cells, and groups of cells, within the tumours (fig. 13).

3 *Cells of the Stroma*—Fibroblasts and endothelial cells, especially of the more malignant tumours, exhibit marked hypertrophy. What is regarded as the sequence of changes in the fibroblast during hypertrophy and degeneration is shown in figs. 26-33. The changes include increase in number of the Golgi bodies (*gb*), followed by their anastomosis to form a network (*ga*), which breaks up during degeneration and forms granules (*gg*). The Golgi apparatus of hypertrophied endothelial cells is similarly enlarged, as shown at p. 572.

4. *Muscular Tissue*.—Penetration of the panniculus carnosus by the tumour cells induces reaction on the part of the muscle fibres. There is an increase in the sarcoplasm (*p*), the nuclei (*n*) increase in numbers—probably as the result of amitosis (M, figs. 35 and 36), the mitochondria (*m*) become more numerous (fig. 10), and the Golgi bodies (*gb*) proliferate, and spread throughout the sarcoplasm (fig. 5 and M, figs. 35 and 36).

5. *Cell Hypertrophy and Malignancy*.—In the more malignant tumours the stroma is more abundant, more cellular in character, and its constituent cells are often hypertrophied. Possibly the tumour cells release some growth-promoting substances, or the products of autolysis of necrotic cells may stimulate the growth of surviving cells.

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*Electrical Responses of Extensor Muscles during Postural  
(Myotatic) Contraction.*

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[PLATE 35.]

LIDDELL and Sherrington (22) have observed that an anti-gravity muscle of decerebrate and thalamic animals responds to relatively slow stretches by active contraction, and it has been concluded that these myotatic reflexes are in large measure responsible for the postural tonus of muscles (29). As the behaviour of the electrical responses during such stretch reflexes has not been previously examined, we have obtained simultaneous mechanical and electrical records of the responses of the quadriceps extensor of cats to stretching, and we propose in this communication to describe the results.



*Method.*—A myograph of high natural frequency (1,600 per second) in the same optical system as the galvanometer has been employed as previously described (13, 15, 17). Two silver pins coated with chloride have been used as leads, one inserted into the tendon, the other inserted into the belly of rectus femoris. The falling table described by Liddell and Sherrington (22) has been used for slow stretches. Cats decerebrated under deep anaesthesia by the trephine method and allowed to recover have been used throughout. All muscles (except the one used) of both hind limbs were immobilised by nerve section and resection of tendons. The knee-jerks studied were usually elicited by tapping the tendon with the finger, but sometimes also by tapping the table, which, although heavy and rigid, yielded probably  $1/40$  mm., thereby stretching the muscle and eliciting the reflex.

*The Relation of the Knee-Jerk to the Motor Twitch.*

The knee-jerk is a fractional form of myotatic reflex (22). The resemblance between the knee-jerk contraction, although reflex, and the twitch contraction evoked by a single-shock stimulus of the motor nerve is sufficient to invite closely detailed comparison between the myograms of the two.

*The Motor Twitch.*—In a recent study (14, 15) employing technique of enhanced accuracy one of us has found five successive features characteristic of maximal isometric twitches of skeletal muscle (frog):—(1) A period of rigidity (50 duration at  $20^{\circ}$ ) previous to shortening (15), (2) an abrupt and almost completely convex (upward) ascent, the point of “inflection” of the curve never being higher than one-twentieth to one-tenth of the total height of the ascent; (3) a flat crest or plateau (Sherrington, 28); (4) a sharp termination of the plateau which has been called the “angle” (14), (5) a descent which is concave upward in outline, suggesting a purely passive process. It has been shown that the more perfect the mechanical and physiological conditions of recording, the more precise is the “angle”, from this it has been inferred that the “angle” represents some fundamental discontinuity in the response of each individual muscle fibre.

In the present experiments we have found that the same features characterise single responses of mammalian muscle (quadriceps femoris as a whole; rectus femoris; biceps; gastrocnemius; and soleus). For accurate observation of the “angle” it is necessary to reduce to a minimum the inactive constituents and frictional resistances within and surrounding the muscle. The “angles” in Sherrington’s (28) and in Liddell and Sherrington’s (21) submaximal responses have a very slight “nose” (see Sherrington’s fig. 6), and are not as precise

as those observed in the frog. We have found that when rectus femoris of a decerebrate cat is freed from its fellow muscles in quadriceps and freed as well from all fascia, skin and other frictional resistances about the tendon, a maximal twitch then showed an "angle" as precise as, if not more precise than, the "angle" observed in the gastrocnemius of a frog. This observation is of importance, since the rectus of a mammal has its fibres parallel, and it therefore removes any remote possibility of the "angle" being associated with the diagonal disposition of the fibres in gastrocnemius.

*The Knee-jerk* --When a knee-jerk is elicited from the isolated rectus, or from quadriceps as a whole, by a sharp tap, the kinetic energy of which is expended on the tendon in 1σ or less (as indicated by the initial upward deflection of the myograph, fig. 2), the mechanical record of the resulting response differs from the mechanical record of the motor twitch in the following ways: (1) The period of rigidity is present but less precise and more difficult of accurate measurement in the knee-jerk, (2) the ascent of the knee-jerk is invariably somewhat concave (upward) initially, the point of inflection to convexity being usually somewhat higher than in the motor twitch (*cf.* 24A); (3) the plateau of the knee-jerk tends to be somewhat greater in duration; (4) the "angle" of the knee-jerk, though often very precise, is definitely less sharp than that of the twitch under comparable conditions; and (5) the curve of relaxation is characterised initially by a slight "nose." A comparison of the duration of the knee-jerk with that of the motor responses, measured from the electrical response to the "angle" (14), has revealed that the duration of the knee-jerk is more variable and on an average is always greater than the duration of the mn. twitch. Thus, in one experiment six knee-jerks of the exposed rectus of a decerebrate cat were recorded, then four responses produced by single stimuli applied to the *uncut* nerve (fig. 4), and finally a few minutes after cutting the anterior crural nerve six mn. twitches were taken. The duration of these responses are summarised in Table I on next page.

It should be pointed out that the motor twitch was less in duration than the knee-jerk contraction despite the progressive cooling which may have occurred as a result of exposure of the muscle. In other experiments we have found the duration of the knee-jerk greater than the twitch by values varying from 5 to nearly 50 per cent. The fact that there is very little irregularity in the duration of the twitch when the uncut nerve is stimulated shows that the variability in the case of the knee-jerk is not due to anything inherent in an intact nerve.

Finally, the electrical response of the knee-jerk is invariably somewhat more

**Table I.—Durations of Motor Nerve Twitches of Decerebrate Cats compared with the Duration of the Knee-jerk (rectus femoris).**

Knee-jerk. (0·001 second).	Motor Twitch. Nerve Cut. (0·001 second).	Motor Twitch. Nerve Uncut. (0·001 second).
44·5	36·0	—
49·0	40·0	—
52·0	40·0	40·0
45·0	37·0	42·0
49·0	38·0	41·0
57·0	37·5	40·0
—	—	—
296·5	228·5	163·0
—	—	—
49·4	38·1	40·8

spread out than that of the mn twitch (*cf* fig. 2 with fig. 3 ; same preparation). The point of inflection of the electrical response is again higher in the knee-jerk than in the twitch.

Myographic records of the knee-jerk have shown that the tap on the tendon may increase the tension of the muscle by 50 to 400 gm in 1 to  $2\sigma$  (fig. 5), *i.e.*, the stimulus is of very brief duration. A knee-jerk, however, may be set up when the tap is much less sharp, its duration being 20 to  $30\sigma$  (fig. 6). The electrical response of a knee-jerk produced by a slow tap is somewhat more spread out than that of a jerk produced by a sharp tap, and in keeping with this the mechanical response is of greater duration and shows a less precise “angle” (fig. 6), while a “quick” knee-jerk always has an angle approaching but never quite reaching the sharpness of the “angle” of a motor twitch. We have interpreted this as indicating that the motor impulses, in the case of the “slow” knee-jerk, have not arrived at the muscle in a synchronous volley, but are somewhat spread out in regard to time. The cause of this lies certainly in the slow tap, *i.e.*, the proprioceptive afferent endings are not all stimulated simultaneously.

We infer, moreover, from the foregoing observations that even in the case of the sharpest tap the volley of centrifugal impulses does not reach the muscle quite synchronously. The less precise period of rigidity, the higher point of inflection, the longer plateau, the less precise “angle,” the “nose,” and finally the increased total duration all favour this interpretation. The variability in the duration of individual knee-jerks occurs quite independently of the size

(i.e., tension developed) in the jerk, while in motor twitches sub-maximal responses are of greater duration than maximal responses, because, as one of us has shown (16), the less the shortening occurring during the response of an individual muscle fibre the greater the duration of the response. We therefore regard the variability in duration of the knee-jerks as due to slight variations in the duration and sharpness of the taps. Gross irregularities in individual taps are automatically recorded in our records, but we have noted slightly varying durations of knee-jerk when the taps, as far as the myograph reveals their nature, are apparently identical. Two factors suggest themselves to account for this. (1) Though the speed and kinetic energy of two taps may be identical, their angles of incidence may differ slightly and so affect different afferent endings, since the resulting wave of increased tension would travel in a slightly different direction, (2) inherent variability of the responses of the central nervous system. The possibility of the staircase effect influencing knee-jerks has been considered, but has been ruled out by having a large number of taps before any were recorded.

Several factors suggest themselves in attempting to account for the asynchronism occurring in response to the sharpest of taps: (1) Conduction time of the wave of increased tension down the muscle. Recent observations by Bethe and Happel (1) indicate in the case of the isolated sartorius of the frog that this may be of the order of  $1\sigma$  per cm. If this value held for quadriceps, the afferent stimuli would be spread over  $\pm 8\sigma$ , but as the muscle is initially stretched it is likely that this is a high estimate. (2) The slightly differing conduction rates in an apparently homogeneous volley of nerve impulses which Erlanger and Gasser (8) have demonstrated by means of the cathode ray oscillograph. This would tend to make the volley of impulses spread out slightly as it travelled up and down the nerve. (3) The duration of the tap, which is seldom less than  $1\sigma$  as contrasted with an absolutely instantaneous stimulus of an inductorium. (4) Variations in the latent time of central nerve cells and synapses which are inherent in physiological as contrasted with mechanical systems. Thus Forbes and Gregg (12) found in the case of the action current of a single reflex twitch of a flexor muscle much the same thing that we have found in the action current of the knee-jerk, viz., that it was somewhat more spread out than the action current of the motor twitch, and they concluded as we have that after passing through the spinal centres the volley of impulses emerged somewhat asynchronously. It is evident, therefore, that the asynchronism in the case of the knee-jerk may not be due entirely to asynchronous stimulation of the afferent endings, for in the flexion reflex they are all stimulated simultaneously

by an induced shock. These results seem comparable with those of Sassa and Sherrington (24A), who had found previously that in the reflex flexor myogram there is a less steep ascent than in the motor twitch. Forbes (10, p. 387) has suggested that the central asynchronism may to some extent be accounted for by the assumption that individual impulses may pass along very different and branching reflex paths, and this may be in large measure the explanation of the slight asynchronism of the volley in the case of the knee-jerk. (5) Finally, there is the possibility of repetitive discharge of individual motoneurones, which, if present, cannot be extensive. (See following communication (17).)

*Electrical responses in the stretch reflex.*

With the silver chloride leads inserted into the detached patellar tendon and belly of rectus muscle of a decerebrate cat, no electrical responses are seen if the muscle is flaccid. When, however, the muscle is extended by a tension of several hundred grammes very small rhythmic oscillations are usually observable, similar to those described in muscle in decerebrate rigidity by many previous authors (Dusser de Barenne, 6; Buytendijk, 2; Einthoven, 7; Forbes and Cattell, 11). If an inhibitory nerve is stimulated under these conditions the string again becomes quiet (Dusser de Barenne, 6; Einthoven, 7), and the muscle may undergo slight relaxation (inhibition of tonus, 26, 27, 7). If, however, the inhibitory nerve is strongly stimulated, a small ipsilateral contraction may occur, which mechanically and electrically shows the rhythm of stimulation. Following this, however, there is a period of complete inhibition marked by complete quiescence of the string (17).

The knee-jerk of rectus femoris is, as we have seen, accompanied by a well-marked but somewhat spread-out action current (*cf.* Jolly, 20; Snyder, 30). Similarly a clonic response such as may readily be elicited by attaching the muscle to a weighted isotonic lever shows clearly defined action currents as shown in fig. 7. In these points we confirm all previous workers in this field (Salamonson, 24; Cobb, 3, and others). When, however, rectus femoris is gradually and uniformly stretched, and its myotatic reflex thus elicited, the string may be almost entirely at rest, showing only the slightest vibration at the height of a reflex response of 4 to 6 kilos. When the stretch reflex is inhibited the string occasionally shows one or two small spikes at the beginning of the inhibition, but even at this time the string is often quiet save for the de Barenne-Buytendijk vibration. We have among our plates a number of responses in which the table did not fall with absolute uniformity. The irregularities of stretching caused in this way document themselves in the

myographic record by a sudden small upward movement which is followed by a hump on the ascent of the record. These sudden irregularities in the stretch of the muscle *invariably give rise to a movement* of the string which varies in size with the extent of the irregularity in the stretch. That this movement of the string is an action current and not a fortuitous deflection caused by shifting of contact is evident, since it always follows at an interval of 6 to  $8\sigma$  after the irregularity (fig. 1), and the hump on the mechanical record follows in turn after the movement of the string. Without an accurate mechanical record it would therefore be impossible to interpret these electrical responses. Irregularities in the fall of the table have been imitated in a number of experiments by tapping the tendon *during the stretching*. These taps give rise to sudden increases in the tension, in every way comparable to knee-jerks, which fuse with the increasing active tension produced by the uniformly increasing stretch. Such taps give rise to action currents again after an interval of 6 to  $8\sigma$ , which are usually much larger than those produced by irregularities in the fall of the table. We therefore regard the irregularities in the falling table and the taps on the tendon as similar and comparable proprioceptive stimuli.

We have seen in the case of the simplest myotatic reflex, the knee-jerk, that the responses of the individual muscle elements are probably never quite in phase. If now we apply this conception to the stretch reflex, it is obvious that here, since the stretch is applied very gradually, as by stretching a muscle 90 mm. long by a further 5 mm. during 3 seconds, there will be a process of gradual "recruitment" of the afferent endings, and the more uniform the stretch the more complete will be the resulting asynchronism of the different stimuli. The galvanometer string under these circumstances may be likened to a Brownian particle under bombardment almost indiscriminately in all directions. Like the Brownian particle, the string only occasionally has a sufficiently large number of action currents impinging upon it simultaneously to produce deflection. However, when the table falls in irregular steps, or when a tap is given, relatively large numbers of afferent endings in the muscle would be stimulated simultaneously, with the result that the corresponding proportion of efferent motoneurons would be stimulated in unison.

In eight experiments we have led off to the galvanometer with two shielded silver chloride leads directly from the anterior crural nerve from which the internal saphenous and nerves to sartorius had been carefully dissected away. The unaided galvanometer string was found capable of detecting the efferent volley of impulses produced by a knee-jerk (figs. 5 and 6). The interval

between the beginning of tap and the beginning of the efferent volley in the nerve in the experiment from which fig. 6 was taken was  $5.2\sigma$ . When taken from the muscle instead of the nerve the interval was on an average  $6.6\sigma$ , which is in agreement with Jolly's (21) determinations. In some records there was also a trace of what appeared to be an afferent volley (*cf.* Jolly, 19). In the case of the stretch reflex the string was absolutely quiet, showing not even the small vibrations which we regularly have observed in the muscle. When the nerve action currents obtained in this way were augmented by a two-valve amplifier which Dr H. S. Gasser very kindly designed for us, the electrical responses of the knee-jerk were rendered clearly visible (fig. 6), but their existence was again questionable in the stretch reflex except when the table fell irregularly. From our observations, however, it is clear that the latency of the stretch reflex may be no greater than the latency of the knee-jerk ( $6\sigma$  to  $7\sigma$ , see figs. 1 and 2). This of course applies only to the most rapidly responding elements in the reflex. It is probable that some of the proprioceptive impulses travel up to the higher centres, becoming eventually "integrated" in the mid-brain with the other proprioceptive impulses from the neck, labyrinth, etc. The latent time of the reflex involving these devious paths is probably indeterminable, as it is unlikely that any two impulses would have exactly the same latency.

So long as the silver chloride leads are not touched during a response by skin or fascia, we have observed no progressive and continuous movement of the string during inhibition or during increase of "tonus," such as have lately been described by Foix and Thévenard (9), and by many early writers. We would concur with Einthoven (7) that these slow potential alterations have nothing whatsoever to do with "tonus," but would suggest that they are due probably entirely to shifting of contact in experiments in which wick electrodes were used. The alteration in demarcation current with different passive tensions, which de Meyer (5), Einthoven (7) and others have described, is with silver chloride leads very slight, and in some experiments was not even demonstrable. We have been inclined, therefore, to disregard it.

### *Discussion.*

The minute and variable character of the electrical responses of muscles in decerebrate rigidity and other tonic manifestations has led many writers (Salomonson (24) and others, see Cobb's review (4)) to the inference that postural reactions, especially when exaggerated, as in decerebrate rigidity and other spastic conditions, are produced by some "contractural" or fixing

mechanism other than the all-or-nothing type of response known to occur in individual muscle fibres when their motor nerve is stimulated. Since Cobb (4) has recently reviewed the latest investigations in this field, it will be unnecessary here to give an historical account of these researches. In this paper has been considered the postural response which gives rise to decerebrate rigidity, and it has been pointed out that when an extensor muscle of a decerebrate preparation is detached from its tendon, if unstretched or if completely inhibited, there is no trace of any electrical response. Furthermore, clearly defined electrical responses occur provided the stimulus for the development of the postural reaction occurs with sufficient suddenness to stimulate a large number of afferent endings approximately simultaneously. Such postural reactions as we have investigated are intelligible in terms of one type of contractile response, viz., the all-or-nothing contraction of individual muscle fibres which has been so thoroughly studied in peripheral nerve-muscle preparations. It would appear from this that one of the great integrative functions of the central nervous system, which makes possible steady sustained responses, is the production of completely asynchronous responses of individual muscle elements.

The relative unfatiguability of postural reactions has been urged by many writers as a difficulty in accepting the all-or-nothing view regarding the origin of posture. Forbes (10, p. 403), however, has suggested that the elements in all probability respond in rotation. In favour of this we have noted in tetanic motor responses of extensor muscles (cats weighing  $\pm 2$  kilos) that tensions as great as 40-50 kilos may sometimes be developed, but that in postural responses the tension developed is seldom greater than 5-6 kilos. In view of the relatively quick fatiguability of the motor response, in which the plateau commences to decline after 8 to 10 seconds of stimulation, and the fact that postural responses, though they may decline somewhat at first, eventually reach a steady height, which may last for 30 minutes or more, it seems unlikely that the same fibres are in activity the entire time.

Since increase in tension appears to be the adequate stimulus for the proprioceptive endings of postural (myotatic) reflexes, shifting of the incidence of tension among the afferent endings suggests itself as a cause of the rotation in response of the muscle elements. It will be recalled that myotatic reflexes are local in their effect (25 and 22), the reflex being confined to the particular portion of muscle stimulated (i.e., stretched). Presumably the smallest locus or reflex unit is the group of motor fibres controlled by one "stretch" afferent-organ. At any given moment in a rigid muscle only a small number



of these afferent-efferent systems are active. One would suppose that when such an afferent ending is stretched, the motor fibres controlled by it would contract. If this contraction relieved the stretch on their own afferent ending, thereby removing their own stimulus, other stretch-afferents would be stimulated as a result of this cessation of support in the first afferent-efferent system. Or if the first system suffered depression, as by autogenous inhibition arising from other types of afferents in the muscle (29), then neighbouring afferent-efferent systems would become active and thus maintain the state of rigidity in the muscle. So the process might be conceived of as progressing indefinitely in endless asynchronous rotation. This, substantially, is the conclusion reached by Forbes (10) on other grounds.

Another point in favour of the all-or-nothing interpretation of myotatic postural responses is the fact that such responses can be very rapidly inhibited by appropriate central stimuli (22). Liddell and Sherrington (23) have pointed out that the speed of inhibition of the crossed extension reflex is of the same order of magnitude as the speed of fall of the myograph following cessation of a motor tetanus, although the commencement of the fall, as one might anticipate, is not a precise "angle." In our experiments we have found that the rate of inhibition of the stretch reflex is similarly rapid. If special sarcoplasmic or red-muscle (18) mechanisms were responsible for sustained tonic reactions, it is improbable that they could be so rapidly inhibited, whereas according to the all-or-nothing interpretation the rapid speed of inhibition follows as a direct corollary of an efficient *central* inhibitory mechanism. Unpublished experiments have shown that the rate of relaxation of red muscle (soleus) of the cat is as much as four to five times slower than that of white muscle (isometric records of mn. stimulation).

### *Summary.*

Simultaneous mechanical and electrical records have been obtained of the responses of quadriceps femoris and rectus femoris of decerebrate cats to various forms of postural (myotatic) reflex, and the following are the chief conclusions :—

(1) The knee-jerk (decerebrate preparation) set up by a tap of less than 1σ duration differs from a motor twitch in five respects :—(a) The point of inflection from concavity to convexity usually occurs higher in the mechanical ascent of a knee-jerk than of a twitch ; (b) the plateau is of longer duration ; (c) the "angle" is less sharp and is followed by "nose" ; (d) the total duration of knee-jerk is greater than the twitch and more variable, (e) the electrical

response always has a higher point of inflection and is more spread out. From this we conclude that the knee-jerk is produced by a somewhat asynchronous volley of impulses.

(2) An extensor muscle of a decerebrate preparation shows no vibration of the de Barenne-Buytendijk type in the string when completely inhibited (Einthoven) or when the tendon is detached so that the muscle is completely flaccid. When the tendon is extended by a force of 100 to 400 or more grammes the vibration occurs.

(3) During a stretch reflex in which 4 to 6 kilos active tension are developed the string may be almost completely quiet save for a slight de Barenne-Buytendijk vibration at the height of the response. If, however, the stretch is applied discontinuously, as when the stretching table falls irregularly or when the tendon is tapped during the stretch, well-defined action currents occur at an interval of 6 to  $7\sigma$  after the irregularity or tap. We conclude from this that the comparative absence of electrical responses in the stretch reflex is due to the complete asynchronism of the afferent stimuli, for when a large number are recruited approximately synchronously an action current invariably occurs.

(4) Since, as Sherrington has pointed out, the exaggerated stretch reflex characteristic of the decerebrate condition is in large measure responsible for the rigidity of the extensor muscles in the decerebrate animal, we have inferred that these sustained postural reactions are produced by asynchronous all-or-nothing contractions of the individual muscle fibres rather than by hypothetical fixing mechanisms.

#### DESCRIPTION OF PLATE 35.

FIG. 1.—Quadriceps femoris of decerebrate cat, showing a myotatic reflex in response to a slow stretch. Muscle initially at 600 gms. tension. A stretch of 3 mm. was given in 1.5 seconds, during which the tendon was tapped. The taps are indicated by abrupt upstrokes in the myograph record. The tap in approximately the middle of the ascent is the most effective, as it is followed after  $7\sigma$  by an action current and characteristic (knee-jerk) contraction, which fuses with the increasing active tension. The perpendicular dropped from the tap on the myograph to the string shows the  $7\sigma$  interval. At the height of the stretch the muscle is inhibited by a weak tetanic stimulus applied to the ipsilateral sciatic nerve for an interval indicated by the lower signal. Rate of inhibitory stimulation (50 per second) indicated by vibrating signal immediately above. Line of zero tension above vibrator. Galvanometer string between myograph and line of zero tension. String tension 9 mm. per m.v. at magnification of 285. Leads two silver pins 0.6 mm. diameter coated with chloride. Myograph frequency 1,600 per second. Fall of the table shown (with considerable initial latency) by sloping line near top of the plate. Time above = 0.02 second. 19 mm. vertical distance = 1 kilo tension. Tendon movement magnified  $80\times$  in all figures.

- FIG. 2.—A knee-jerk (quadriceps) of a decerebrate preparation caused by striking the table to which the preparation was fixed. The action current (from muscle) in this figure follows at an interval of  $6.6\sigma$  after the beginning of the tap. "1" in all figures = point of inflection.
- FIG. 3.—Motor twitch of same preparation as fig. 2 taken a few minutes after cutting the nerve. Note the greater brevity of the electrical response as contrasted with fig. 2.
- FIG. 4.—Two responses of rectus femoris produced by stimulating the *uncut* anterior crural nerve. The twitch-like character of the response is shown, but it is followed after an interval of approximately  $55\sigma$  by central after-discharge; leads from the muscle.
- FIG. 5.—Knee-jerk of quadriceps; galvanometer leads taken from the anterior crural nerve. The afferent volley is not seen, but the efferent volley of impulses is clearly visible, following at an interval of  $5.2\sigma$  after the tap. In other responses of this preparation the interval between the tap and the beginning of the action current varied from  $5.0$  to  $5.5\sigma$ . The tip of the myograph used in this experiment vibrated at approximately 500 per second.
- FIG. 6.—A "slow" knee-jerk; leads from the anterior crural nerve with electron-tube amplification. The beginning of the "spike" occurs at  $5.5\sigma$  after the beginning of the tap. The extraneous movement of the string during the response is probably not physiological. Time above =  $0.04$  second.
- FIG. 7.—A clonic response of quadriceps at 13 per second elicited by attaching the muscle to an isotonic lever weighted by 1 kilo.

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Kg

2

1

0

FIG. 1.

Kg

1

0

FIG. 2.

FIG. 4.

Kg

2

1

0

FIG. 3.

FIG. 5.

FIG. 6.

FIG. 7.



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*Observations on Spinal and Decerebrate Knee-Jerks, with Special Reference to their Inhibition by Single Break-Shocks.*

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[PLATES 36 AND 37.]

The present communication deals with certain differences observed in knee-jerk reflexes when elicited from decerebrate animals (i) with intact spinal cord, and (ii) with the spinal cord separated from the higher centres by section in the thoracic region, and further with their different behaviour to inhibition produced by single break-shocks. Sherrington was the first to show that the knee-jerk was inhibitable like other spinal reflexes (32, 35), and the work of Jolly (23), of Snyder (39), of Dodge (9) and of Viets (40A) contributed further to the recognition of the jerk as a true reflex phenomenon.

*Method.*—The isometric myograph of high vibration frequency, previously described (16, 18, 20, 21) has been used throughout with a string galvanometer (new Cambridge pattern) arranged in the same optical system. As before, the reflex has been elicited by a finger tap on the tendon or by tapping the table with the finger or light hammer (Waller, 41). Control records such as that

shown in fig. 1, A, have shown that the taps so elicited are constant (in height) within 5 per cent. The animals used have been cats, decerebrated at the inter-collicular level by the trephine method under deep anaesthesia, and allowed to recover. When they are made "spinal," the level of transection has been usually between the last thoracic and the first lumbar root. The section was made under anaesthesia sometimes before, sometimes after, decerebration. In some cases the preparations have been used "decerebrate," and after some hours have been made spinal. In one case a decapitate preparation was used. In a small number of cases the cord had been severed by aseptic operation a few days previously. For inhibiting the jerk a single break induction shock has been used, delivered in all cases to an ipsilateral afferent nerve. The leads from the muscle for recording the electrical responses were two silver pins, coated with chloride, one in the tendon, the other in the belly of rectus femoris.

During experiments with the "spinal" preparation, it has been our practice to slacken the tension off the muscle between observations. In our hands, long continued tension on the muscle (or a higher tension, as is necessary in the spinal preparation for elicitation of the jerk) produces after a time enfeeblement of the response.

The coreless Berne inductorium, which was used throughout, did not set up "supramaximal" responses ("multiple firing") (14, 36) in mammalian motor nerve when the coil distance was as small as 1 to 2 cm (cf. 19) with a 2 volt accumulator in the primary circuit. For inhibition of spinal jerks a single stimulus with the secondary at 12 to 18 cm. coil distance was adequate to produce complete inhibition of the jerk. For inhibition of the decerebrate jerk a much stronger shock was required (3 to 5 cm.). We found the hamstring nerve more effective in producing inhibition than the sciatic. In one experiment at 18 cm. coil distance the hamstring nerve was as effective as the sciatic nerve at 12 cm. coil distance. During experimentation, the preparations lay always in the left lateral position. The right limb was used throughout.

#### I.—A COMPARISON OF THE KNEE-JERK IN SPINAL AND DECEREBRATE ANIMALS.

In the previous communication (21) it was pointed out that the decerebrate knee-jerk differed slightly from the motor twitch in five respects, and it was inferred from these small differences that whereas the motor twitch is set up by synchronous volley of impulses, the decerebrate knee-jerk is caused by a volley of impulses in which the individual impulses are somewhat out of phase.

The duration of the decerebrate knee-jerk was found always somewhat greater and less constant than the duration of the twitch, being from 5 to 50 per cent. longer.

In the present investigation we have compared the mechanical and electrical characteristics of the *spinal* knee-jerk with those of the *decerebrate* knee-jerk. The five characteristics which distinguish the motor twitch from the decerebrate knee-jerk (the less precise "period of rigidity," the higher point of inflection, the less sharp "angle," the longer plateau and the "nose") also distinguish the spinal knee-jerk from the twitch; the higher point of inflection is, however, a somewhat less constant feature. In two important mechanical features, the spinal knee-jerk differs from the decerebrate.

(1) The *duration* (*i.e.*, the interval between the beginning of the electrical response and the "angle" (17) of the spinal knee-jerk ( $80-130\sigma$ ) is two to three times longer than the duration of the motor twitch ( $37-40\sigma$ ) or decerebrate knee-jerk  $43-60\sigma$ ). (Fig. 7; cf. fig. 1, Plate 36 (decerebrate), with fig. 2 (spinal).)

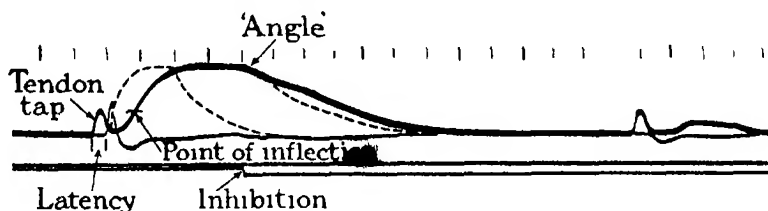


FIG. 7.—Knee-jerk of spinal preparation taken on "fast" plate to show the interval between the tap and the action current—in this case  $8.4\sigma$ . The point of inflection on the mechanical ascent is shown, as well as the "angle". The duration of the response is approximately  $80\sigma$  as contrasted with a motor twitch shown as a dotted line of  $38\sigma$ . An inhibitory stimulus is applied at the moment indicated. The ipsilateral twitch (20) resulting from this has modified the relaxation, the unmodified course of the relaxation is shown by a dotted line. The second response to a tap on the tendon is very small, owing to the long-lasting effect of the single inhibitory stimulus. The duration of this second response is approximately  $50\sigma$ . Time intervals,  $0.02$  sec.

Despite the greater duration of the spinal knee-jerk the "angle" (17, 21) may be, and usually is, surprisingly sharp (fig. 2, A), showing that the majority of the individual muscle fibres have ceased contracting approximately simultaneously. Occasionally in a deteriorating decerebrate preparation the knee-jerks become long and spinal in character. In one preparation we noted that this was associated with a failure of the crossed extension reflex, and we have been inclined to regard it, therefore, as an example of a "spinal-decerebrate animal," according to the usage of Prof. Graham Brown (5, 6).

(2) The curve of relaxation from the spinal knee-jerk though not as rapid



as that of a twitch (see fig. 7) is generally smooth and (except at the beginning) concave (upward) in shape; while the decerebrate knee-jerk, in a preparation with good rigidity, invariably shows a "hump" (fig. 2, D, Plate 36, 1st response) at about half-relaxation which, following Sherrington (35, A), we have regarded as a manifestation of the shortening reaction, and is possibly due to certain elements responding to the stimulus of the sudden stretch caused by the subsidence of the jerk. The "hump" appears, therefore, to be a myotatic appendage of the decerebrate jerk.

Other features of the spinal and decerebrate knee-jerks are :—

(3) Electrically the differences between the spinal and decerebrate knee-jerk are not marked. In three\* of five preparations in which we obtained decerebrate and spinal knee-jerks from the same animal the action current was slightly more spread out ( $1-2\sigma$ ) in the spinal jerk than in the decerebrate (*cf.* action currents in figs. 1 and 2, Plate 36), and the small diphasic portion of the excursion was considerably longer, *i.e.*, the string remained below the base-line longer in the spinal than in the decerebrate response, and in a few spinal records there was a very slight vibration of the string during this period (fig. 2, B, Plate 36, second response).† Apart from these very slight and often inconstant differences, the string record of a spinal knee-jerk is indistinguishable from that of a similar decerebrate response

(4) The latency, *i.e.*, the interval between the commencement of the tap and the first movement of the string, is, in our experience, 10 to 30 per cent. greater in the spinal preparation than in the decerebrate, when elicited by comparable afferent stimuli. Thus in the figs. 1 and 2 (Plate 36) values taken at random from any four spinal responses are :  $8.2\sigma$ ,  $8.5\sigma$ ,  $9.5\sigma$ ,  $9.0\sigma$ , while in the decerebrate response they are :  $6.0\sigma$ ,  $6.2\sigma$ ,  $7.0\sigma$ ,  $5.8\sigma$ . In fig. 7, in which is shown the record of a spinal knee-jerk taken on a "fast" plate, the latency is  $8.4\sigma$ . In other preparations the decerebrate latency has varied from  $5.8\sigma$  to  $7.3\sigma$  with an average of  $6.6\sigma$  and the spinal latency from  $7.0\sigma$  to  $10\sigma$  with an average of  $8.8\sigma$ . The cause of this increase in latency may lie either in an increased latency of the afferent ending due in some way to the mechanical condition prevailing in the "atonic" muscle or it may be of central origin. Though the latter appears *a priori* more probable, we have no evidence to settle the point.

\* In these three the leading-off electrodes were allowed to remain in place during the spinal transection.

† This downward movement of the string was, we believe, not due to movement of the electrodes.

The fact that the latency or the knee-jerk is less in the decerebrate preparation than in the spinal is in keeping with earlier workers (*see* Jolly, 23) who insisted that even in the presence of the higher centres the knee-jerk is wholly a spinal reflex. The differences between the spinal and decerebrate jerks already enumerated suggest that the higher centres exert their control over such spinal reflexes as the knee-jerk by modifying (through inhibition, *see* below) the *condition* of the (spinal) anterior horn cells or internuncial neurones directly involved in the reflex.

(5) In its difficulty of elicitation, the spinal knee-jerk contrasts markedly with the decerebrate. Other things being equal, the initial tension of the muscle must be from 100 to 300 per cent. greater in the spinal condition for the knee-jerk to be elicited by a comparable tap on the tendon. Under the same initial tension (*e.g.*, 200 gm.) of the muscle the tap, in order to be effective in the spinal preparation, must be several times more ample than suffices in the decerebrate (as judged by the initial upstroke of the myograph—*cf.* figs. 1 and 2, Plate 36). In this connection we have found the threshold for elicitation of the jerk much higher in a spinal preparation in which the spinal transection was made before decerebrating, than in a preparation which had been allowed fully to recover from the anæsthetic (and the decerebration) and which was made spinal several hours later (with anæsthetic). We can offer no adequate explanation for this observation.

(6) The interval between the electrical response and active shortening ("period of rigidity," etc (18)) is in the majority of our records greater in the spinal jerks (fig. 2) than in the decerebrate (fig. 1). This appears so even when the muscle is stretched initially. Its cause may be associated in some way with the fact that in "tonic" muscle the tension is presumably maintained by a certain proportion of actively contracting elements (21), while in atonic muscle it is maintained by the passive stretch of inactive constituents.

#### *Interpretation of Differences.*

The two most fundamental differences between the spinal and decerebrate jerks lie in their respective durations and their curves of relaxation. We have inferred that the decerebrate jerk is probably produced by a single (or double) and somewhat asynchronous volley of impulses from the motoneurones. Our mechanical stimulus lasts only  $1-2\sigma$  (21), and it is unlikely, considering the refractory period of nerve fibres, that more than one volley of centripetal impulses is set up, or if more than one, *e.g.* by repetitive firing of the *afferent* organs under

a constant stimulus,\* then the interval between the successive impulses in the majority of our records would appear to be at any rate as brief as  $1-2\sigma$ . The longer duration of the spinal jerk raises the question whether the knee-jerk of a spinal animal can likewise be regarded as having been produced by an asynchronous but single volley, or whether it results from repetitive discharge of central motoneurones, i.e., a brief tetanus.

*Duration.*—In favour of a single asynchronous volley it might be urged that when released from higher control, the impulses produced by the afferent stimuli may wander through branching nerve-cell systems ("delay paths"; Forbes, 12) possibly affecting eventually a larger number of spinal motoneurones so that on emergence the earliest of the motor impulses are separated from the latest by as much as  $40$  to  $50\sigma$ , and that this accounts for increased duration of the response. This explanation is made improbable (1) by the abrupt commencement of the spinal knee-jerk, the point of inflection (21) as already mentioned, being low and often scarcely distinguishable from a motor twitch. (2) The "angle" of a spinal knee-jerk even when the response lasts as long as  $130\sigma$  (as contrasted with  $40\sigma$  the duration of a mn. twitch) is very sharp (fig. 2, A, Plate 36)—proving that the majority of elements cease contracting simultaneously (17, 18), which would be impossible if the responses of the individual elements were spread out, for when a response such as a stretch reflex (in which the elements respond asynchronously) is abruptly inhibited, one never observes a sharp "angle" (28, 37, 21). Nor is it observed in the inhibition of after-discharge or rebound (29) or the crossed extensor reflex (20), where the elements also respond asynchronously.

A second possibility of the longer duration of the spinal knee-jerk is that it is of peripheral origin (17) due to the high initial tension that has been necessary in our experience for elicitation of the spinal jerk. Control records, however, have been taken of motor muscle-nerve twitches from the same and even higher initial tensions. Increase in initial passive tension does not increase the duration of the twitch by more than one-fifth, while double the duration is demanded if the results in the reflexes are to be explained by peripheral causes.

Again, one might interpret the longer duration of the spinal knee-jerk as due to red muscle fibres responding rather than white. Though we have no convincing evidence to disprove this possibility, two small points might be

\* Genuine "after-discharge" as an after-phenomenon separate from possible repetitive firing of an afferent muscle organ under constant stimulus has not, so far as we are aware, been demonstrated.

urged against it: (1) the electrical response of red muscle (soleus) has been examined in unpublished experiments, and was found to be approximately four times longer in duration than that of white muscle, which is not the case in the electrical responses of the spinal jerk, and (2) the "angle" of red muscle in our experience is never so sharp as the "angles" which we have observed in spinal knee-jerks.

A fourth interpretation of the enhanced duration of the spinal jerk above that of a twitch is that the individual motoneurons giving rise to it respond repetitively, *i.e.*, give rise to a short tetanus. Against this is the almost complete absence of any evidence of repetition in the electrical record. But if the successive impulses follow each other at very brief intervals, and if the individual motoneurons do not repeat at exactly the same intervals—and it is highly improbable that they do so (Forbes and Olmsted, 15, Adrian, 1), the string, owing to the resulting inevitable asynchronism, would, after the first response, show only the slightest de Barenne-Buytendijk vibration (10, 7), and this latter it occasionally does show (see above).<sup>\*</sup> Moreover, if a *tetanus* of four or five volleys occurred, each volley separated by an average of  $6\sigma$ , the shape of the mechanical response so produced would be precisely that which is observed in the spinal knee-jerk—*i.e.*, it would rise abruptly and reach a flat plateau which would terminate in a sharp angle, since under these circumstances *all* the elements would begin and cease contracting at approximately the same time. In fig. 3 (Plate 37) are reproduced the mechanical responses of several such brief tetani obtained from frog muscle. It will be noted in the (fused) response of fig. 3, A (Plate 37), that when the second (maximal)<sup>†</sup> action current of the fused response occurs  $4\sigma$  after the first one, *i.e.*, when the string is returning to rest at nearly maximum velocity, the second electrical response (even though caused by a synchronous volley) shows itself in the string merely as a slight "hesitation" in its descent. If this second volley were slightly spread out, even by as little as  $0.4\sigma$ , its only effect would be to give the first response an apparently longer duration, *i.e.*, delay its return to the base line.

Added to the evidence from the direct analysis of motor tetani, there are the

<sup>\*</sup> Jolly (23, p. 82) occasionally observed a repetition of the electrical responses, but at an interval of 32 to  $37\sigma$ . This he attributes to repetition blows of his undamped "tapping" hammer causing successive volleys of afferent stimuli.

<sup>†</sup> If it were not maximal—and the second response to a "supramaximal" stimulus frequently is not maximal—the mechanical response would show it by a smothered "angle" and a more or less prominent "nose," on the curve of relaxation. These characteristics are the expression of asynchronous cessation of contraction of individual fibres.

observations of Sherrington (36) and of Sassa and Sherrington (31A) that a reflex flexor response to a single shock, which (as judged myographically) is made up of repetitive responses, shows a flat plateau and characteristic "angle" (see their figures). It might, however, happen for example that half the motoneurons fired off twice and the other half four times. Under such circumstances the "angle" of the resulting composite response would be smothered, as has been observed in root stimulation when one root supplying gastrocnemius was stimulated twice at the same time that the other was stimulated but once. On this basis one could account for a small proportion of spinal knee-jerks which showed no "angle" at all (see first jerk in fig. 4, Plate 37, and compare with Sherrington's fig. 6, upper curve). We have observed knee-jerks without "angles" most frequently in preparations in which the cord had been cut aseptically some days before recording the knee-jerks.

Finally, in favour of the repetitive (or tetanic) nature of the spinal jerk is the fact that when emerging from an inhibition (see below) the spinal jerk may be only  $40\sigma$  in duration (i.e., the same as a motor twitch) (fig 2) suggesting that the first motoneurons to "awake" from an inhibition are able to respond only *once* to the afferent volley resulting from a tap. In other words, *a spinal knee-jerk when under inhibition—that is, when deprived of its repetitive character—is indistinguishable in duration from a decerebrate knee-jerk.*

In frog muscle, a tetanus of two responses separated by 4 to  $8\sigma$  is usually 40 to 50 per cent. longer than a single twitch, but a tetanus of *three* similarly-timed impulses may be only 60 to 80 per cent. longer than the twitch. As the spinal knee-jerk is 100 to 250 per cent. longer than a twitch, it would appear, since the responses of mammalian skeletal muscle do not differ in any fundamental way from that of the frog (18, 19), that the spinal knee-jerk is produced by a tetanus composed of at least 4 to 8 volleys. Observations on short motor tetani (2 to 5 volleys at 200 to 250 per second) of quadriceps (cat) are entirely in keeping with these observations on the frog. Thus, in one experiment on a cat's quadriceps, in which the successive responses of a tetanus were separated by  $\pm 6\sigma$ , the twitch had a duration of  $42\sigma$  (initial tension 200 gm.); while a tetanus of two responses lasted  $65\sigma$ , one of three responses  $81\sigma$ , and of five responses  $105\sigma$ .

In our experiments we have no direct evidence of the actual *rate* of discharge of individual motoneurons producing the tetanus responsible for the spinal jerk. However, the string does not come to rest completely from its diphasic excursion (in which small vibrations sometimes occur) for 40 to  $50\sigma$  after

its first deflection, and since 4 to 8 volleys appear to have reached the muscle in this time, this places the rate of discharge between 100 and 200 per sec. (*cf.* Adrian, 1 : Gasser and Newcomber, 22).

*Relaxation.*—The differently formed curves of relaxation in the two types of knee-jerk demand consideration. The rate of fall of the first third to a-half of the relaxation may be almost as rapid in the decerebrate jerks as in a motor twitch, though the curve is always somewhat less concave than the motor twitch. The corresponding portion of the relaxation of a spinal jerk, on the other hand, has a slope (in responses of the same tensions) often only about half as steep as the slope of a motor response. This suggests that while the majority of contractile elements must have ceased contracting at the “angle” in a spinal jerk a certain small proportion outlast the “angle” and so modify the curve of relaxation.

In the decerebrate jerk, as already pointed out, increase in the “tonus” of the muscle (shortening reaction) shows itself at about half-relaxation (fig. 1, B and D, Plate 36), by a somewhat large “hump” in the descent of the curve. This in the past (35, A) has been referred to as tonic “after-discharge” of the decerebrate knee-jerk, and as already mentioned (p. 592) we look upon it as a myotatic appendage caused by the stretch incident upon the sudden relaxation of the jerk. Being postural (*i.e.*, myotatic) in character it is readily inhibitable, and if a single-break shock is applied to the ipsilateral sciatic nerve 10 $\sigma$  after a tap (fig. 1, D, Plate 36, second response) a pure twitch-like knee-jerk occurs, unmodified in its relaxation by this tonic after-discharge. Fig. 1, B, Plate 36, and fig. 9, show inhibition of “tonus” quite apart from the inhibition of tonic after-discharge.

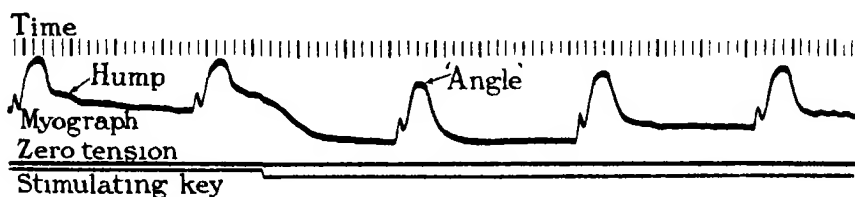


FIG. 9.—Relaxation of tonus (decerebrate preparation) following single inhibitory break-shock. Electrical responses not recorded. Note that after inhibition the “angles” are sharper and the duration of the responses somewhat more brief. The durations in this record lie between 40 and 50 $\sigma$ . Time, 0.02 sec.

It is clear from these observations that we must distinguish two varieties of after-discharge in association with the knee-jerk. (1) The repetitive “volley-fire” after-discharge, which is characteristic of the spinal knee-jerk (see fig. 7),

and characteristic also of a reflex flexor response to a single stimulus, is probably an after-phenomenon of *spinal* motoneurones. This repetitive type of after-discharge is often almost entirely absent (fig. 9) from the knee-jerk of a decerebrate preparation in good condition, being presumably cut short by central inhibition from higher levels of the nervous system (mid-brain, &c.).

(2) Tonic or postural after-discharge characteristic of the cessation of the kinetic phase of the decerebrate knee-jerk is recognised by the "hump," the slow decline in tension of which merges gradually into the base line. (See fig. 1, B, second response) It is dependent upon connection of the cord with the mid-brain and higher centres, and is an index of the postural activity (stretch reflex) of the muscle of a decerebrate preparation. It may be due to the activity of other units than those responding in the kinetic phase of the knee-jerk. This type of after-discharge has not been observed in our records of "acute" spinal knee-jerks. In one aseptic spinal preparation (cord cut five days before experiment), a small amount of inhibitable spinal "tonus" was present at the beginning of the experiment. (See fig. 4.)

The behaviour of the string in spinal knee-jerks strengthens our previous conclusion (20) that when the discharge is asynchronous the string vibrations can in the nature of things provide no real evidence of the rate of discharge of individual motoneurones, and it also shows that absence of string movement is not necessarily an adequate proof of absence of repetitive after-discharge.

## II.—INHIBITION OF THE KNEE-JERK BY SINGLE BREAK SHOCKS.

### A.—The "Spinal" Knee-jerk.

When a single break-shock inhibition of adequate intensity is applied by an ipsilateral afferent nerve in a spinal animal during a series of knee-jerks, there is a marked effect within 1 to 2σ (fig. 10) on the amplitude of the reflex response,

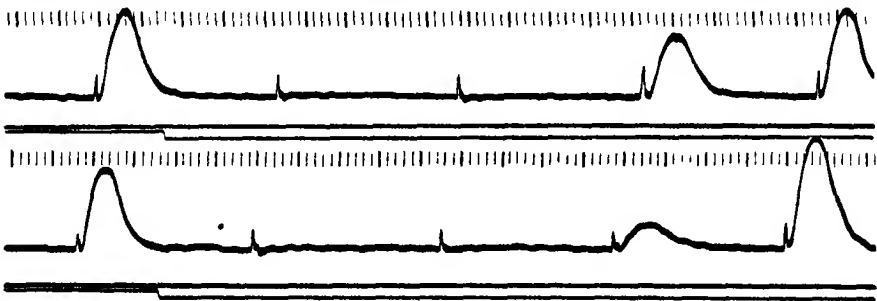


FIG. 10.—Spinal preparation showing complete inhibition of the knee-jerk for a period of 1 sec. In the lower record the taps are smaller than in the upper. Strength of inhibition 8 cm. Time above = 0.02 sec. String record not shown (see Plate 1).

in spite of the fact that there may be at that time as yet no diminution in "tone" or variation in the mechanical stimulus. The reflex response is profoundly affected by the inhibition, even to complete obliteration, and then slowly recovers (*conf.* Viets (40A)). The rate of recovery follows a gradual curve and it has been possible to express our results graphically (fig. 8).

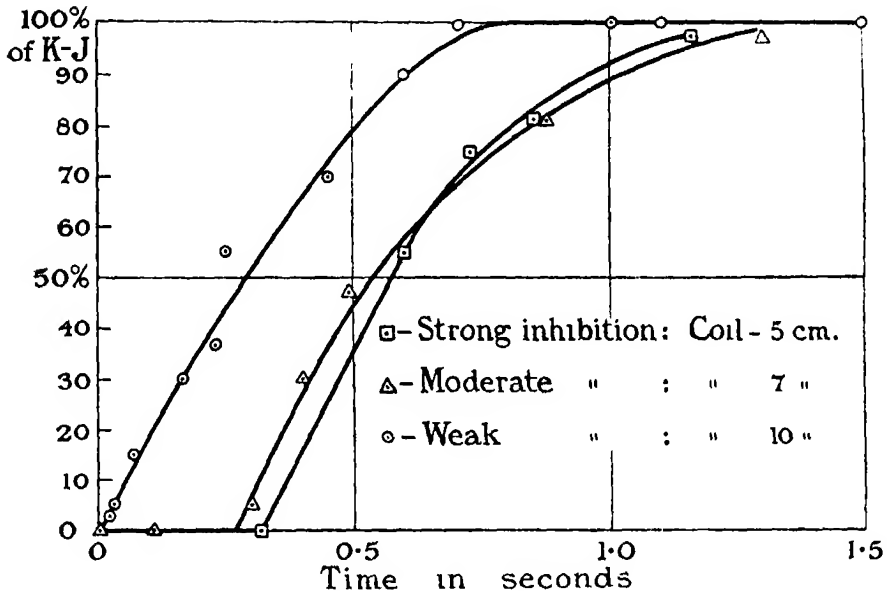


FIG. 8.—Recovery curve of the knee-jerk of a spinal animal after a single inhibitory break-shock applied to the sciatic nerve. Ordinate = height of response expressed as percentage height (*i.e.*, tension) of a "normal" response to a series of equal taps. Abscissæ = time in seconds after inhibition. The first curve (circles) shows the recovery from a "weak" inhibition (*i.e.*, coil 10 cm.); at this strength only when the tap and the inhibition were simultaneous was inhibition complete. The second curve (triangles) shows a stronger inhibition (coil 7 cm.). After which inhibition was "complete" for 0.3 sec. A stronger inhibition (coil 5 cm.) is shown in the third curve (squares), but as recovery follows almost the same curve as the previous one, this suggests that the previous strength of stimulation was *maximal*. All the responses shown in this graph were obtained from the same preparation within a period of thirty minutes.

The ordinates represent the reflex response after inhibition to a series of equal taps, expressed as the percentage height (*i.e.*, tension) of a normal response (fig. 8). The abscissæ represent time after the inhibitory stimulus in fractions of a second, *i.e.*, the moment of inhibitory stimulation is taken as zero on the time scale, and the amplitude of the knee-jerk is plotted at various time-intervals after the inhibitory stimulus. The resulting curve, therefore,



represents at any instant the number of central nerve-units which have "awakened" from the inhibition. It will be seen that the spinal reflex centres show a gradual recovery following a single break shock inhibition. Those centres which have recovered do not in our experience show any alteration in their latent period to the mechanical stimulus. The process of recovery is less rapid when the inhibitory shock is strong than when it is weak. We have noted, however, that after a certain limiting strength of stimulation the duration of depression of the reflex is not further increased by increase of strength of the inhibitory stimuli (fig. 8). Thus, in one experiment full recovery had occurred in 1.5 sec. after an inhibitory stimulus at 9 mm. coil distance; similar recovery occurred in 1.5 to 1.6 sec. at 5 cm. coil distance. We have interpreted this on the all-or-none principle as indicating that the *maximal* value of inhibitory stimulus had been reached. In several experiments with such maximal inhibitory stimuli we observed *complete* inhibition (fig. 10) of six successive knee-jerks recurring at 0.33-sec. intervals, *i.e.*, the complete depression of the reflex lasted for 2 secs.

It is of interest to note that the duration of the spinal jerk, when the reflex centres are recovering from inhibition, is shortened and approaches the duration of a decerebrate knee-jerk. Thus in fig. 2 the durations of jerks in the upper row following inhibition are  $40\sigma$  and  $53\sigma$  respectively. This justifies the argument that in the spinal jerk, the reflex centres are responding repetitively and asynchronously, and that after inhibition, neurones recover gradually, according to the degree of depressed activity into which the inhibition had thrown them, those which recover first being able to respond but once. It is also in keeping with the observation of Liddell and Sherrington (29) that repetitive after-discharge is more easily inhibited than the "body" of a reflex response.

#### B.—The "decerebrate" knee-jerk.

In our experience, the inhibition of the "decerebrate" knee-jerk is a more complex phenomenon. It soon became apparent that stronger stimuli are needed to produce inhibition, *e.g.*, to have the secondary coil at least 4 to 6 cm. distance instead of 12 to 18 cm. While it is not at all difficult to inhibit the tonic element (11, 34) in the muscle (fig. 1. C, Plate 36, and fig. 9) and with stronger stimuli enhance the tone (rebound) after preliminary inhibition (fig. 6, Plate 37), it is difficult even with strong stimuli to show any effect on the knee-jerk itself unless the tendon is tapped within 1 to  $40\sigma$  after delivery of a maximal inhibitory shock. With weaker stimuli, inhibition is effective only when it occurs within  $\pm 2\sigma$  of the tap. Our experiments on decerebrate preparations

do not provide a graph showing the exact time course of recovery, for recovery occurs rapidly, that is, the spinal centres when in connection with the medulla and mid-brain seem to be protected, as it were, against inhibition, *i.e.*, in the decerebrate preparation the reflex inhibition provoked by a given stimulus is, as regard the centres of the knee-jerk, less strong and more evanescent than in the spinal condition. Inhibition of the jerk, when it does occur, seems to be almost an "all-or-none" phenomenon, and is rapidly recovered from. We have noticed, however, that though the amplitude of the jerk is affected with such difficulty by inhibitory break shocks, the tonic after-discharge, which ensues during the decline of the curve is easily diminished (fig. 1, B and D, Plate 36, fig. 9) (as judged by the horizontal width of the muscle curves at levels below half relaxation) and that following the diminution there is a gradual return to normal during a period of 0.5 to 1 secs.

In a few of our decerebrate preparations, it was found that the first knee-jerk after inhibition escaped any inhibitory effect on its amplitude, but that the *second* jerk (0.33 sec. later) and sometimes the third were considerably diminished in amplitude (fig 6, Plate 37). The cause underlying this effect has yet to be elucidated. The amplitude of the mechanical stimulus was the same in both cases, and the decrease in initial tension of the muscle due to the inhibitory action on the posture of the muscle, *i.e.*, to its diminution in "tone" amounted only to 50 to 100 gms., representing in one case a fall from 200 gms. of absolute tension to 125 gms. It may be that this slight decrease in postural activity of the muscle affects the kinetic activity of the knee-jerk, as by rendering the afferent organs in the muscle less susceptible to the same absolute degree of mechanical stimulus. Or it may be that the inhibitory stimuli, which were strong and visibly affected the fore limbs, passed not directly to the motoneurone involved in the jerk, but by a path through the mid-brain. This view does not necessarily conflict with that of Liddell and Sherrington (26, 29) and others that inhibition occurs far "down-stream" in the reflex arc, probably at the final motoneurone, but merely suggests that in some reflexes the inhibitory impulses may reach the anterior horn cells both directly and *viâ* the mid-brain.

In two preparations which showed pronounced extensor rigidity in which the inhibitory effect showed itself most markedly on the amplitude of the *second* knee-jerk, the inhibition was not without effect upon the *shape* of the first jerk. Thus, in fig. 6, Plate 37. the first (decerebrate) jerk following the inhibition is only 40σ in duration as contrasted with 62σ, the duration of its predecessor; it has a lower point of inflection than the preceding jerk, and a sharper "angle." These observations suggest that although the amplitude

of the jerk has not been affected the repetitive after-discharge—which (as distinguished from tonic after-discharge) is of spinal-origin—has been diminished by the inhibition. Moreover, repetitive after-discharge has been shown (29) to be more easily inhibited than responses to motoneurons when under constant stimulation. In addition to mechanical differences, *the first jerk after inhibition in these preparations has a much larger electrical response* (fig. 6, Plate 37, 3rd response). From a study of such knee-jerks as these, it is possible to say with reference to the electrical response that when two knee-jerks occur of the same height (*i.e.*, tension) the electrical response will be the *largest* in the jerk which mechanically has the lowest point of inflection and the sharpest “angle.” There would appear to be but one possible inference from these observations: namely, that the effect of the inhibition has been to make the reflex volley more synchronous, as well as to abolish any tendency toward repetitive after-discharge. This lends further evidence to the view that even in the decerebrate knee-jerks the volley of impulses is normally asynchronous (21). The shorter duration of this first response can be interpreted as due to the result of the more perfect synchronization or to the cutting down a small amount of repetitive volley fire such as is found in greater amount in the spinal knee-jerk. It would appear likely that both factors are involved.

A further point of interest is that after inhibition the characters of the second and third knee-jerks of these decerebrate preparations, namely, the longer duration and gradual recovery (see fig. 6A), are suggestive of a spinal condition. In other words, at the time when the second and third knee-jerks are elicited, the preparation appears to have become functionally spinal—as a result of inhibition of the centres in the mid-brain—and is giving the responses of a purely spinal character consequent on spinal inhibition. By the time of the fourth jerk the mid-brain centres have recovered from inhibition, and the preparation appears functionally decerebrate once more.

#### *Discussion.*

The observations on the inhibition of the knee-jerk (which is admittedly a reflex phenomenon\*) make possible certain inferences concerning the nature of the central inhibitory process. In the knee-jerk one has elicited a reflex by a “natural” stimulus, and the balance of evidence indicates that the sharp tap sets up but a *single* volley of afferent impulses, for, as Adrian and Cooper (2) (see also Forbes, Cambell and Williams (13)), have pointed out, afferent proprioceptive impulses continue in a muscle nerve only as long as the mechanical

\* But *cf.* 3, 40, 24 and 30.

stimulus is applied (in our knee-jerks this was 1-2 $\sigma$ ). Since the spinal flexor (reflex) response to a single induction shock is tetanic in nature (31A) (36), the repetitive nature of the spinal knee-jerk does not necessarily indicate repetitive afferent stimulation.

The inhibition of the knee-jerk by a single break-shock, therefore, provides evidence that a *single* inhibitory volley of impulses may inhibit a single excitatory volley of impulses. This occurs both in the spinal and decerebrate condition, but, as has been shown, the single inhibition is more effective in the spinal condition. In fact the long-lasting depression (1 to 3 secs.) of the reflex resulting from *one* inhibitory shock in the spinal animal would seem to preclude the possibility that this inhibition is a condition resulting from a Wedensky type of interference (8), for it continues to exist quite independently of any excitatory process. Our observations, moreover, demand that the inhibitory state should be a long-lasting phenomenon, brought about by the rapid formation and gradual dissipation of actively depressant qualities ("inhibitory substance"), and would, therefore, harmonise with the view of inhibition put forward by Sherrington (38).

The greater difficulty which we have experienced in inhibiting the decerebrate knee-jerks suggests that when in the presence of the higher centres afferent inhibitory stimuli are in large measure, as it were, "long-circuited" up the cord to the higher centres, while in the absence of these higher integrating organs, the afferent stimuli pursue more readily the phylogenetically older spinal paths directly to the anterior horn cells. In favour of this is the fact that the inhibitory stimulus in the decerebrate animal may show a second large effect on the knee-jerk 200 to 300 $\sigma$  after the immediate effect of the stimulus appears to have passed away (fig. 6).

The tetanic nature of the spinal knee-jerk, as contrasted with the twitch-like character of the decerebrate knee-jerk, together with the fact that when emerging from an inhibition the spinal knee-jerk is also twitch-like, and with the fact that the decerebrate knee-jerk seems to resist ipsilateral inhibition, suggests in accordance with established conceptions that the higher centres of the nervous system exert a control on the lower spinal centres through the constant "play" of impulses which can raise or lower (*i.e.*, by inhibition) the threshold of excitation of the spinal centres.

#### *Summary.*

Simultaneous mechanical torsion-wire myograph and electrical records have been obtained of knee-jerks in decerebrate and spinal preparations, and an analysis has been made of their normal characteristics as well as of the charac-

teristics of their inhibition by single break shocks. The following are the chief conclusions.

1. The spinal knee-jerk differs from the decerebrate knee-jerk in five important respects: (1) The mechanical response of the spinal jerk is two to three times longer in duration than that of the decerebrate jerk (or the motor twitch). (2) The curve of relaxation of the spinal jerk is characterised by a less steep but smooth decline, while the decerebrate jerk relaxes at first with a speed approaching that of a motor twitch until it reaches approximately "half-relaxation," when a "hump" appears (postural after-discharge). (3) The electrical response, though apparently single, tends to be more prolonged in the spinal knee-jerk. (4) The latency, *i.e.*, the interval between the tap and the electrical response, is greater in the spinal preparations ( $8.8\sigma$ ) than in the decerebrate ( $6.6\sigma$ ). (5) The spinal jerk is more difficult to elicit than the decerebrate.

2. We infer that the shortest spinal knee-jerk observed is produced by a repetitive discharge of at least 4 to 8 volleys of impulses recurring somewhat asynchronously at 100 to 200 per sec. The decerebrate jerk on the other hand usually partakes more of the character of a motor twitch, though occasionally it too appears to be made up of two or even three volleys of impulses.

3. Both the spinal and the decerebrate knee-jerks may be inhibited by a single appropriately-timed break-shock applied to an ipsilateral afferent nerve. Thus a single excitatory volley of impulses (evoked by a tap  $1-2\sigma$  in duration) can be inhibited by a single inhibitory volley.

4. The jerk of a spinal preparation, however, is much more readily inhibited than a decerebrate, the effect of a single break-shock lasting 1 to 3 seconds. A curve is reproduced presenting the rate of recovery from a single inhibition.

5. When recovering from an inhibition the spinal knee-jerk has the same duration as a twitch or a decerebrate knee-jerk. This favours the interpretation that normally the spinal knee-jerk is tetanic in nature, since the inhibitory stimulus appears to have inhibited repetitive after-discharge.

6. In two knee-jerks of equal size (*i.e.*, tension) but differing shape, the largest electrical response is observed in the jerk in which the point of inflection of the mechanical ascent is the lowest and in which the "angle" is the sharpest. We have interpreted this as indicating that in the latter case the (first) volley of impulses producing the response is the more synchronous of the two. According to this criterion a knee-jerk recovering from inhibition appears in the majority of cases to be more synchronously produced than an uninhibited knee-jerk.

Our thanks are due to Prof. Sir Charles Sherrington for his invaluable advice and criticism during the course of our researches in the past year, and to the Christopher Welch Trustees for defraying the expenses of photographic plates.

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#### DESCRIPTION OF PLATES.

In all figures, except fig. 3 (frog), 19 mm. vertical distance on the plates = 1,000 gms. tension, and time is indicated at the top of all figs. (except 3, B and 3, C) in intervals of 0.02 sec.

#### PLATE 36.

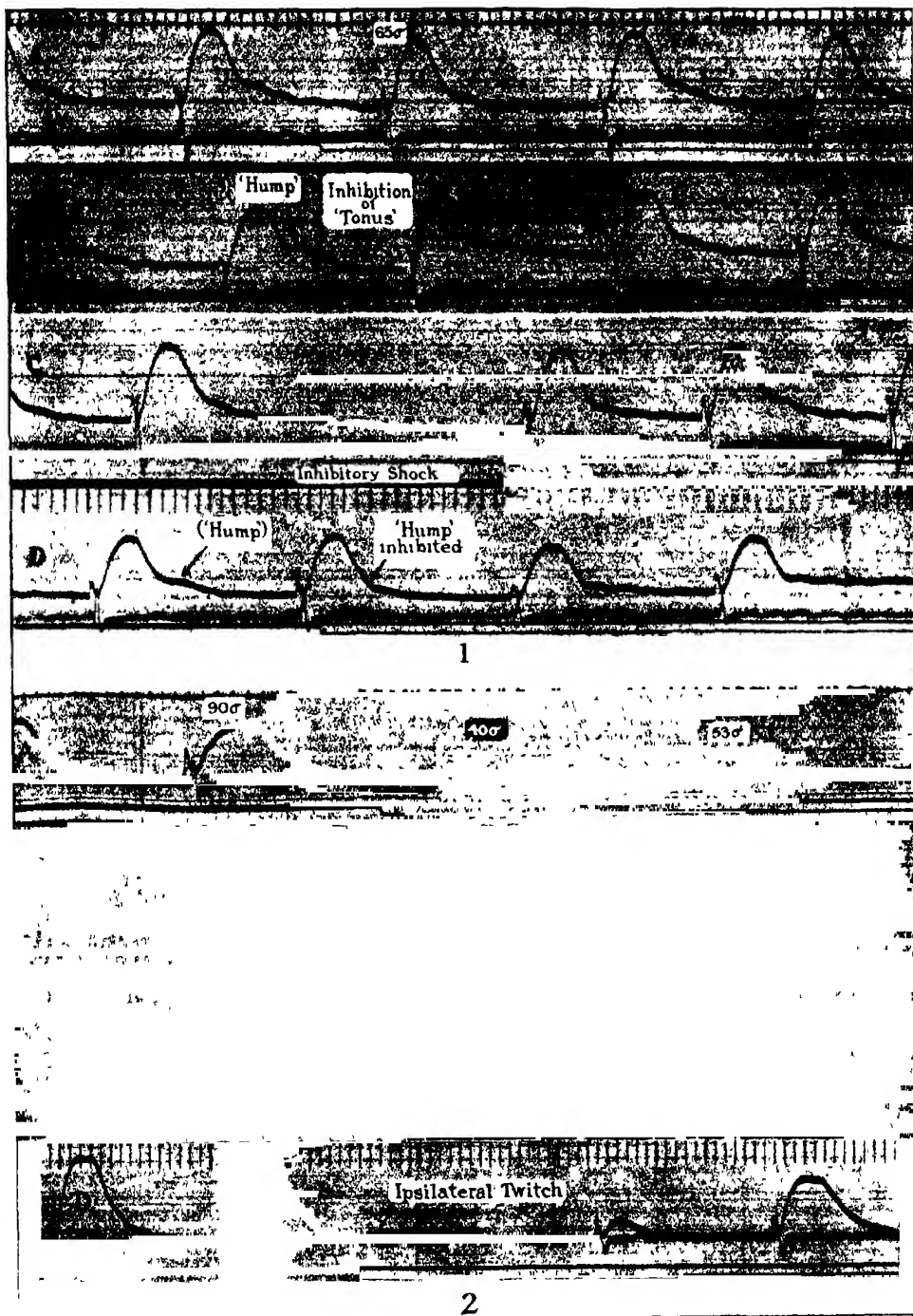
FIG. 1.—Simultaneous electrical and mechanical records of knee-jerks of a decerebrate preparation elicited by successive finger taps. The taps are to be seen in the myographic record as initial upstrokes preceding the response. An inhibitory break-shock is applied to the ipsilateral (hamstring) nerve at the point indicated by the lower signal ( $\frac{1}{4}$  the distance down the plate). Line of zero tension above signal, and above that the galvanometer string: duration of mechanical responses 60 to 70 $\sigma$ . Time at top of records = 0.02 sec. Tendon movement magnified  $\times 84$  (in all figures). String tension = 7 mm. per m.v. at magnification of 295.

- A.—Interval between inhibition and tap 0.10 sec. Effect of inhibition very slight; note "tonic" after-discharge ("hump") towards the end of relaxation in each response. Coil distance = 13 cm.  
 B.—Interval between inhibition and ensuing tap = 0.14 sec. No effect on response, but note the decrease in "tonus" resulting from inhibition. Coil distance 0.  
 C.—Interval between tap and inhibition 0.14 sec. Inhibition of jerk complete. Coil distance = 0.  
 D.—Inhibition occurs 40 $\sigma$  after tap. Note inhibition of tonic after-discharge as evidenced by comparing the first response with the second. Taps smaller in this record than in the preceding.

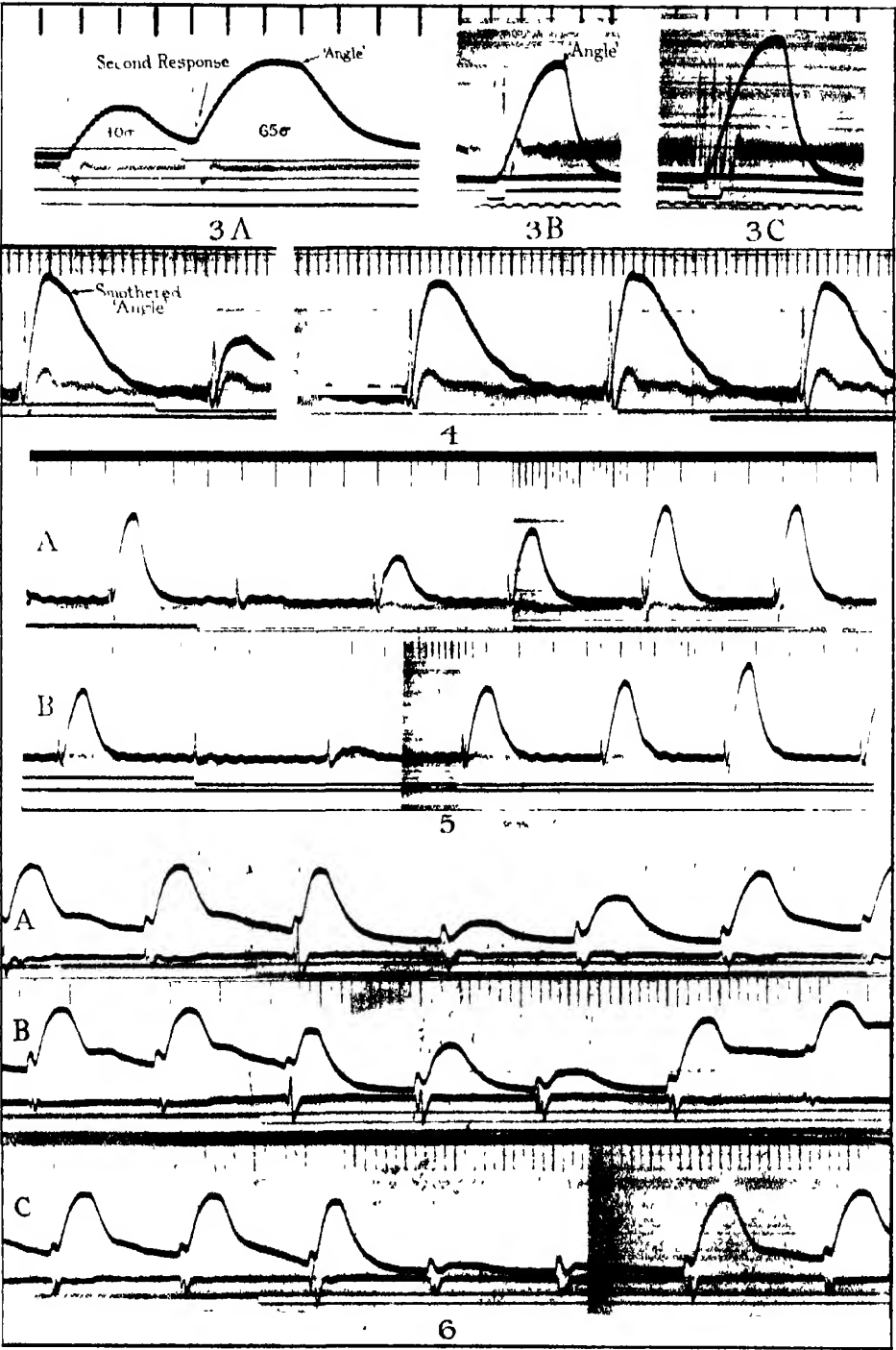
FIG. 2.—Spinal knee-jerks of the same preparation as shown in fig. 1 taken an hour later after spinal section. Average duration of "normal" mechanical response 90 $\sigma$ . Strength of inhibition = 13 cm. coil distance in all responses (applied to hamstring nerve). Other conditions as in fig. 1. String tension 7 mm. per m.v.

- A. Two normal responses (first one partly cut off) followed by single inhibition. The duration of the first response emerging from the inhibition is 40 $\sigma$ , or approximately that of a (single volley) motor twitch.

The duration of the second response is longer (53 $\sigma$ ), which suggests beginning of repetitive "firing." Note also the diminution in the electrical responses.







B. Same with complete inhibition of first response and 60 per cent. recovery of second occurring  $630\sigma$  after inhibition. Compare the electrical responses and note latencies.

C. Same with inhibition occurring  $30\sigma$  after the tap.

D. Same showing *pari passu* recovery of three successive jerks. Slower plate.

There is in this record a small ipsilateral reflex twitch caused by the inhibitory stimulus. Same preparation as that from which fig. 7 was taken.

PLATE 37.

FIG. 3.—“Supramaximal” responses (frog muscle) showing the increased duration of the fused response above that of the twitch when the interval between the fused responses is of very brief duration (4 to  $10\sigma$ ).

A. Single response (duration  $40\sigma$ ) followed by double response (duration  $65\sigma$ ), the second stimulus being a supramaximal “make” of a coreless coil. The interval between the two action currents of the fused response is approximately  $4\sigma$ . Frequency of myograph 500 per sec.  $T = 24^\circ$ . Time above =  $0.02$  sec.

B. A tetanus of three responses. Note the precise angle.  $T = 15^\circ$ . Time above =  $0.04$  sec.

C. A tetanus of 5 responses at an average interval of 10, resulting from a double stimulus applied to a (dry ?) nerve.  $T = 16^\circ$ . Duration of single response of the same preparation =  $51\sigma$ ; that of tetanus  $135\sigma$ . Time above =  $0.04$  sec.

FIG. 4.—A series of spinal knee-jerks (“aseptic” spinal preparation) in which several mechanical responses (1 and 4) do not show an “angle.” This can be interpreted as evidence of asynchronous cessation of contraction in the individual responses, due presumably to an unequal number of repetitive “discharges” reaching individual muscle fibres.

FIG. 5.—Spinal knee-jerks showing gradual recovery. In B the inhibitory stimulus and the tap appear to be almost exactly simultaneous.

FIG. 6.—Knee-jerks from a decerebrate preparation with marked extensor rigidity, showing inhibitory stimuli which were more effective on a second knee-jerk than on the first immediately following the stimulus. In C the duration of the first knee-jerk after inhibition is  $40\sigma$  as contrasted with the normal jerk of  $63\sigma$ . Also, the action current is much larger in this response than in the normal response preceding it, suggesting absence of repetitive firing as well as more perfect synchronization. Though a decerebrate preparation, the recovery of the second and third knee-jerks in A is gradual and distinctly spinal in character, suggesting that the mid-brain centres have been inhibited, making the preparation for a brief interval functionally spinal. In B the string record has been “touched” to make it clearer.



**OBITUARY NOTICES**  
**OF**  
**FELLOWS DECEASED.**

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## WILLIAM BOTTING HEMSLEY--1843 1924.

WILLIAM BOTTING HEMSLEY, born at East Hoathly near Uckfield, on December 29, 1843, belonged to a Sussex yeoman family well-known in gardening circles. A delicate child, Hemsley had to be taught at home, under medical advice he was kept as much as possible in the open air. This led him to take, at an early age, a keen interest in the plants in his father's nurseries where, when lessons ceased, he began work as a "garden boy." Here he showed an aptitude for botanical study, which attracted attention outside his family, and led to his admission to Kew before he was seventeen.

Hemsley entered the Royal Gardens, as an "improver," on September 6, 1860; the recommendation that warranted his early selection was addressed to Sir William Hooker, then the Director, by Mrs Eardley Hall, daughter of Mr. William Borrer, of Henfield, the well-known British botanist. Six months later, Hemsley was posted to the herbarium for temporary duty, under the personal supervision of the Curator, Mr Allan Black; this continued till autumn, 1862, when Hemsley resumed outdoor duty, now as a "young gardener."

The opportunity for intercourse with the talented Black, whose early death in 1866 was deplored by contemporary botanists, had some influence on Hemsley's career. Black, who had himself begun work as an apprentice in a nursery garden, approved Hemsley's wish to become a botanist. But Black did not limit his encouragement to commendation of what Hemsley knew: he pointed out what Hemsley had still to learn. For this, through life, Hemsley remained grateful, we may attribute what Hemsley did, on reverting to the gardens, to advice given by Black. Hemsley did not lay aside altogether the systematic interests that had hitherto engrossed his attention. But he now subordinated these to interests more general. He tried to become sufficiently familiar with modern languages to be able to read German and French; he thought it better to write correct Latin descriptions of plants he knew, than to give all his leisure to the identification of unfamiliar species.

Accident enabled Hemsley to do more than he could have hoped. When he resumed garden work he made the acquaintance of a youth, five years older than himself, who had just arrived. The decision of this stranger to become a gardener had been the consequence of interests awakened while studying botany at his University; his object in entering Kew was to compare English methods with those he had been taught. In this case divergence of aim added strength to community of interest. The lads became fast friends; for over two years they lodged together, and spent their spare time in

methodical study. Before Dr. Edmund Goeze went home to win, as he did, the esteem of botanists and gardeners, as head of the Botanic Garden at Greifswald in Prussia. Hemsley had gained a German undergraduate's knowledge of vegetable physiology and morphology, while Goeze had gained an English gardener's knowledge of plants. Both had become sound French scholars, Hemsley could speak and write German as readily as Goeze could write and speak English.

When they had shared rooms rather less than a year, Hemsley was recalled to the herbarium on special duty. The President of the Linnean Society, Mr. George Bentham, then working there as a volunteer, had just completed the first volume of the '*Flora Australiensis*' for Sir William Hooker's series of colonial floras. But Bentham was at the same time collaborating with Dr. (afterwards Sir Joseph) Hooker on their '*Genera Plantarum*,' the first instalment of which appeared in 1862; in the preface to the Australian volume, published in 1863, there is a reference by Bentham to "Mr. W. Hemsley, a young but able assistant, who has carefully checked my proofs with the Herbarium." A few months later Hemsley showed what progress he had made with his studies in general botany; he gained the first prize at an examination in botany, open to all competitors, held by the Society of Arts in 1864.

At the time that this examination took place the staff of the herbarium at Kew was being reorganised; the emoluments of its members were so inadequate that when Black left in 1864 to assume charge of a public garden in India, Government amalgamated the curatorship and the librarianship, and appointed the librarian, Prof. D. Oliver, to a new "keepership of the herbarium and library." Reduction of staff did not mean diminution of work; Hemsley's engagement as "temporary assistant" was therefore continued. In May, 1865, a permanent post became vacant, and in June, 1865, on the recommendation of Dr. Hooker, Hemsley was appointed by Government a "herbarium clerk" in succession to Mr. A. Smith, deceased.

Soon after official approval had been given to the proposal by Sir William Hooker for a series of colonial floras, Prof. John Lindley resolved to prepare an economic botanical lexicon. In collaboration with Mr. T. Moore, of Chelsea, he proceeded to carry out his scheme: Black, at Kew, was a leading contributor to the first volume of their useful work, the '*Treasury of Botany*.' When Black left for India, his place was taken by Smith; when Smith died, the editors asked Hemsley to write the articles left unfinished by his predecessor. These articles, published in 1866, were Hemsley's earliest contributions to botanical literature; they were followed in 1867 by a paper in the '*Journal of Botany*' on the distribution of British Umbelliferae, and in 1868 by another in the '*Journal of Travel and Natural History*' on the vegetable productions of Abyssinia. Before the Abyssinian paper appeared Hemsley fell ill. Advised

as a boy to follow an outdoor occupation, he had, as a young man, exchanged this for sedentary work. His physique proved unequal to the strain: in September, 1867, he was obliged to resign the public service and return home.

Hemsley's illness, though so serious as to necessitate retirement, did not affect his botanical ardour. At first too unwell to do garden-work, he spent much of his time out of doors and devoted a round of the seasons to an intensive study of the vegetation of his native county; in 1868 he contributed notes on the flora of Sussex to the '*Journal of Botany*.' As soon as his strength allowed he resumed work in his father's nurseries and during 1869-71 he published nothing. This was not because he had abandoned botanical pursuits; on the contrary, his scanty leisure had been given to two sustained tasks. In the field he still studied the Sussex flora, at home he prepared an authorised adaptation to English needs of the '*Manuel de l'Amateur des Jardins*,' for which French garden-lovers had been indebted to Professor Decaisne and M. Naudin. Hemsley's field studies were embodied in a summary analysis of the flowering plants and ferns of Sussex, an abstract of which was given in the 1872 '*Report of the British Association*.' The adaptation of the French '*Manuel*' took the form of a '*Handbook of Hardy Trees, Shrubs and Herbaceous Plants*,' often consulted still by British gardeners.

When these two works had been completed, Hemsley left home to collaborate at Rothamsted with Mr. (afterwards Sir John) Bennett Lawes in an investigation of the mixed herbage of permanent meadows; no medical objection was taken to a step that ensured occupation out of doors and at the same time offered a change of air and scene. Soon after Hemsley reached Rothamsted in 1872 his analysis of the Sussex flora was published in full in the '*Journal of Botany*'; shortly before Hemsley left Rothamsted in 1873 his '*Handbook*' was issued. His stay at Rothamsted helped to promote his recovery; though his health was still far from satisfactory, and throughout his life was never more than indifferent, he now felt able to return to Kew to work, as a visitor, in the herbarium.

Hemsley's study of the vegetation of Sussex during 1868-71 seems to have led to a desire, not unnatural in a Sussex man, to write a '*Flora*' of his county. During 1874 he worked at Kew with this object and published in 1875, as an appendix to the '*Journal of Botany*,' an outline of the flora of Sussex, which led to his being elected an associate of the Linnean Society. In 1876 he supplied a number of additions and corrections to this outline and dealt, in another paper in the '*Journal of Botany*,' with the botanical results of his work at Rothamsted during 1872-73. He was also able to assist the medical school of St Mary's Hospital to tide over an emergency by serving, during the session of 1876, as "lecturer on botany."

Hemsley's '*Handbook*' was given a welcome which showed that it met a need. This success had its own consequences. A widespread demand for botanical notes by Hemsley came from the editors of English and Belgian



gardening serials ; during 1874–83 Hemsley wrote some two hundred of these—usually brief, always good. In 1875 he was appointed “Lindley librarian” by the Royal Horticultural Society ; in 1877 he had to supervise a re-issue of his ‘Handbook’ ; in 1878 he contributed three important chapters to Moore’s new edition of Thompson’s ‘Gardener’s Assistant.’

The judgment of Hemsley’s botanical and horticultural contemporaries was noted by contemporary zoologists. The editors of the ‘*Biologia Centrali-Americana*,’ a work devoted to the natural history of the territories between the southern frontier of the United States and the Isthmus of Panama, asked Hemsley to prepare the botanical section. If botany could congratulate Messrs. Godman and Salvin because Hemsley consented, these naturalists were soon to earn the thanks of botany for having enticed Hemsley away from the lanes and downs of Sussex to more remote and less familiar fields. Hemsley began his preliminary estimate of the available material in 1877. In 1878, in 1879, and in 1880, he published three fascicles of ‘Diagnoses’ of new or imperfectly described Mexican and Central American plants, and made, meanwhile, such progress with the main work that the editors were able to issue the first instalment of the botanical section of the ‘*Biologia*’ in September, 1879. Nor was this task, which occupied much of Hemsley’s time during the next nine years, the only impediment to the preparation by him of an English county flora. He undertook to assist Dr J. E. T. Aitchison in describing, from Aitchison’s material, the flora of the Kuram Valley in Afghanistan, their results were published in two papers in the Linnean Society’s ‘*Journal*’ in 1880 and in 1882. Before the second Afghan paper appeared, Hemsley had written, for Miss Marianne North, a descriptive catalogue of the “paintings of plants and their homes,” executed by her and exhibited in a gallery at Kew, erected at her expense for presentation, with its contents, to the nation. This catalogue, published by Miss North in 1882, was accepted by the Stationery Office, without modification, as an ‘Official Guide to the North Gallery.’

Hemsley’s work on the plants of the Kuram Valley brought about his reappointment to the herbarium staff at Kew, in 1883, as “assistant for India” ; acceptance of this post, then only a part-time one, did not involve the abandonment of existing obligations or prevent him from incurring new ones. The preparation of the descriptive catalogue of Miss North’s pictures had necessitated close study of the salient facts of plant distribution and plant association in many parts of the globe ; the amount of exact information condensed in this small work, as unassuming in appearance as it is valuable in substance, may explain invitations received by Hemsley in 1883 to supply to ‘*Nature*’ a note on the botanical results of the voyage of “H.M.S. Challenger,” and to write the official report on the flowering plants collected by the naturalists attached to that expedition. Hemsley began work on this ‘Report’ in 1884 ; it was published in 1885. It deals with the material collected in all the islands visited

by the vessel, and includes a chapter on "drift seeds" as well as an introductory essay on "insular floras." This report at once gained for Hemsley a reputation abroad comparable with that which, ten years earlier, his Sussex studies had brought him at home.

While Hemsley was "assistant for India," the '*Biologia Centrali-Americana*' made the first claim on his private time: so sustained was his progress that the editors were able to issue the final instalment of their botanical section in October, 1888. That section occupies four volumes of text and one of plates: the text includes an introduction and an appendix dealing with geographical botany. Its completion was followed in 1889 by Hemsley's election as a Fellow of the Royal Society and a little later by honorary membership of the "Antonio Alzate" Natural History Society of Mexico. Next in order came the claim of an undertaking to prepare, in collaboration with Mr. F. B. Forbes, an '*Index Florae Sinensis*,' the first instalment of which was published in the Linnæan Society's '*Journal*' in 1886. Failing health prevented Forbes from taking a personal share in this task after that instalment appeared; Hemsley was thus left to labour single-handed. Nevertheless, the first of the three volumes which Forbes had enabled the Society to set apart for the '*Index*' was completed in 1888, and the opening instalment of the second volume was printed in 1889. A year later Hemsley became once more a permanent civil servant and could only continue the '*Index*' during non-official time: his progress was now less rapid; the second volume was not completed till 1894; the third and last was not completed till 1905. This work has placed systematic botany under an obligation at least as great as that created by the botanical section of the '*Biologia Centrali-Americana*.' Nor did Hemsley's Mexican and Chinese studies absorb all his attention: he continued the work on "insular floras," begun while preparing his "Challenger" report. In that volume he had dealt with the vegetation of the Bermudas, St. Paul's Rocks, Fernando Noronha, Ascension, St. Helena, South Trinidad, Tristan da Cunha, the Crozets, Kerguelen, the Macdonald Group, Amsterdam and St. Paul Islands, Juan Fernandez, Masafuera, San Ambrosio, San Felix, the South-Eastern Moluccas and the Admiralty Islands. While assistant for India he dealt in his private time with Diego Garcia and South Georgia in 1886; with the Kermadecs in 1888; with the Kuriles, Christmas Island and the Keelings in 1890. As a member of the permanent staff at Kew he dealt officially with the Solomon Islands, the Revilla Gigedos and Lord Howe Island in 1891; with the Tonga Archipelago in 1894; with the Galapagos in 1895, in 1898 and in 1900; with the new vegetation of Krakatoa in 1903. During 1894-96 he contributed to '*Science Progress*' a series of seven articles on insular floras; his interest in this subject remained a dominant one to the end of his active career.

Hemsley's official duties as assistant for India brought him into touch with the survey work of the Calcutta Botanic Garden, carried out by Dr. (afterwards

Sir George) King : one result was a paper by Hemsley on Perak plants, published in the 'Journal of Botany' in 1887. They led to renewed association with Aitchison, whom Hemsley assisted in working out the botanical results of the Afghan Delimitation Commission, published in the Linnean Society's 'Transactions' in 1888. They involved collaboration with Sir Henry Collett in a paper on the botany of Burma, published in the Linnean 'Journal' in 1890, and with Mr. J. H. Lace in a paper on the botany of Baluchistan, published in the Linnean 'Journal' in 1891. But the contributions to Indian botany with which Hemsley's name is thus associated, represent only a small part of the service rendered by him to India. For seven years he gave inestimable assistance to the official Indian botanists who were then, with a self-effacement as marked as his own, devoting all their energies to the provision of material for use by Sir Joseph Hooker while preparing the 'Flora of British India.'

Before the Baluchistan paper had appeared, Oliver vacated the keepership which had been created for him in 1864. Government appointed Mr. J. G. Baker, principal assistant in the herbarium at Kew, to the post Oliver had held. The director, Mr. (afterwards Sir William) Thiselton-Dyer, recommended the appointment of Hemsley in succession to Baker : this recommendation was approved, and in 1890 Hemsley was readmitted to the branch of the civil service from which he had been invalided in 1867. As "principal assistant" Hemsley now took his share in contributing to the 'Kew Bulletin' and to 'Hooker's Icones Plantarum,' both edited at Kew. In 1896, at Hemsley's request, the Linnean Society accorded him transfer from the status of Associate to that of a Fellow. At the close of 1898, Sir Joseph Hooker dedicated to Hemsley the volume of the 'Botanical Magazine' for the year. A fortnight later Baker's tenure of the keepership came to a close and, as from January 13, 1899, Government appointed Hemsley "keeper of the herbarium and library." During 1903-4 Sir Joseph Hooker, who had edited the 'Botanical Magazine' since 1865, associated Hemsley with himself as co-editor. In this capacity Hemsley wrote a history of the 'Magazine' since its foundation in 1787; this history was issued by the publishers in 1906, together with an index to the plants figured during 1787-1904. In 1908, when Hemsley's public service was about to cease, he was elected a corresponding member of the Deutsche Botanische Gesellschaft : on reaching the prescribed age, Hemsley followed Oliver and Baker into retirement on December 28, 1908.

Though it was no easy task to replace predecessors so eminent, Hemsley filled the keepership with distinction. The simple courtesy of his welcome to friends and to strangers alike ; the unwearied kindness with which he treated requests for help ; the unaffected modesty with which his wide knowledge was placed at the disposal of others, endeared him both to colleagues and to visitors. His linguistic gifts enabled him to make workers from continental countries feel as much at home in the herbarium as he did those from outlying British

dependencies and from the United States. But he made it no secret that, in his case, demission of the multifarious and exacting duties of the keepership had compensations : it enabled him to resume studies, the prosecution of which he had, for a time, been compelled to forgo. Taking up residence at Twickenham, so as to be within easy reach of Kew, his attendance at the herbarium during the next six years was almost as regular as it had been since 1890

The most important of the studies now taken up by Hemsley related to New Zealand. Two years before Hemsley retired his friend, Mr. T. F. Cheeseman, had completed the '*Manual of the New Zealand Flora*,' to the preparation of which Cheeseman had devoted many years. While the '*Manual*' was passing through the press, Cheeseman decided that it would be useful if, as a complement to that work, there could be issued a series of '*Illustrations of the New Zealand Flora*'. Many types of New Zealand species are preserved at Kew, where Sir Joseph Hooker wrote, for his father's series of colonial floras, a '*Handbook of the New Zealand Flora*,' published during 1864-67 : the work now projected must therefore, Cheeseman felt, so far at least as the figures were concerned, be prepared where Hooker's types are available. It was accordingly arranged that the drawings for the new work be executed at Kew by Miss M. Smith, under the personal supervision of Hemsley, as keeper of the herbarium. After his retirement, Hemsley continued to exercise this supervision, and was now able to assist Cheeseman in connection with the '*Illustrations*' in other ways. This task occupied much of Hemsley's attention till 1912, when the last of the material left for Wellington, where the work was published in 1914.

Hemsley returned, after retirement, to the study of Chinese plants, largely with the object of assisting Prof. (afterwards Sir Isaac) Bayley Balfour, who had, since 1904 been giving much of his leisure to the extension of what had been accomplished by Hemsley himself in the '*Index Florae Sinensis*' ; the last contribution by Hemsley to Chinese botany appeared in 1914. But at the same time the expedition of Mr. J. C. F. Fryer to the Indian Ocean, the zoological results of which were published in the Linnean '*Transactions*' in 1911, had led Hemsley to revert to the study of insular floras and to begin the preparation of a flora of Aldabra. In 1915 Hemsley supplemented his history of the '*Botanical Magazine*' by publishing a valuable account of the work of the well known botanical artist, Mr. W. H. Fitch. This was not the only indication Hemsley gave, after retirement, that his sympathy with gardencraft was as keen as ever. Between the time that his '*Handbook*' was issued and the date of his appointment as assistant for India, Hemsley had contributed botanical notes to gardening journals at an average rate of some twenty each year. While he held his Indian assistantship this average fell to seven ; when he rejoined the home civil service it fell to four ; after his retirement the number once more rose. For the preparation of such notes

Hemsley was well qualified. His early work for the 'Treasury of Botany' taught him to satisfy the requirements of an editor so competent as Lindley. He had command of a concise yet easy style. He was intimately acquainted with the needs of intelligent cultivators. He had enjoyed an unusual advantage: while studying general botany during 1862-63, he had learned to regard the living plant from a standpoint familiar now, but one rarely adopted in this country until a decade after Hemsley won his botany prize in 1864. This may account for the fact that many of the notes contributed by Hemsley to gardening journals deal with applications to cultural practice of physiological principles. However this may be, the thanks of the science he served for his systematic studies of Central American, Chinese, Indian and Insular plants were not more sincere than the thanks of the craft in which he had been trained originally, for the many useful botanical notes written on its behalf.

In 1909 the Royal Horticultural Society, shortly after Hemsley's retirement, recognised the services he had rendered gardeners, since 1873, by the award of the Victoria Medal of Honour. In 1910 the Royal Society of New South Wales, remembering all he had done for botany since he checked Bentham's Australian proof-sheets in 1863, elected him an honorary Fellow. In 1913, while the two volumes of Cheeseman's 'Illustrations' were passing through the press, the New Zealand Institute showed its appreciation of Hemsley's share in their preparation by electing him an honorary Member. In 1913, also, the University of Aberdeen conferred on Hemsley the honorary degree of LL.D

While, in 1915, Hemsley was at work on the flora of Aldabra, his visits to the herbarium at Kew became less regular. Signs of failing health, at first more disquieting to his friends than to himself, became evident, and in 1917 he was advised to move from the Thames valley to a more bracing environment. He left Twickenham for Broadstairs, but the improvement in health for which his friends hoped was not realised. Gradual but steady decay of his physical powers prevented Hemsley from revisiting Kew, and compelled him to seek the aid of the permanent herbarium staff in completing his work on the plants of Aldabra. That help was readily accorded and the 'Flora of Aldabra,' published in the 'Kew Bulletin' in 1919, was Hemsley's last important contribution to botanical literature. With intellect unimpaired, but during the latest years of his life physically helpless, Hemsley died at Broadstairs on October 7, 1924, leaving to surviving fellow-labourers the memory of a loyal colleague and a faithful friend, whose sound judgment and capacity for taking pains had enabled him to render permanent service to systematic botany; whose firsthand knowledge of its needs had enabled him to give instant help to garden-craft.

The recognition by horticulture of that help has, incidentally, supplied us with an appreciation of Hemsley's career written for gardeners by a botanist

able to speak from intimate acquaintance with Hemsley's scientific work. Hemsley's return to Kew, as a visitor in the herbarium, coincided with the commencement there of the distinguished services of Sir William Thiselton-Dyer, assistant director of the Royal Botanic Gardens, 1875-85, director, 1885-1905. When the Royal Horticultural Society added Hemsley's name to the list of recipients of the Victoria Medal of Honour, his former chief said, in the 'Gardeners' Chronicle' :--" We have laboured together in many undertakings of which he has borne the principal burden. And, looking back on a past which must reach to some forty years, I recognise throughout the same retiring and unpushful disposition and modest diffidence which I well remember when we first met. These pages will meet the eye of many young men. They may well take note of the career of a man without backing, and with nothing to start with but character and love of knowledge, whose early education must have been slender, and who throughout life had to struggle with ill-health. Yet he taught himself German from a colleague, and to write French and Latin fluently, and in the most unassuming way, but with unwearying industry and courage, attained scientific distinction, and, within the limits of his science, a reputation which is cosmopolitan."

D. P

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## SIR WILLIAM A. HERDMAN—1858–1924.\*

WILLIAM ABBOTT HERDMAN was born in Edinburgh on September 8, 1858, and died in London on July 21, 1924, after a strenuous life, in which he had attained great distinction, not only as a zoologist and oceanographer, but as a man of unbounded activity in the councils of those engaged in the organisation and promotion of science.

Educated at the Edinburgh Academy, he showed an early indication of the bent of his mind by the foundation of a school Field Naturalists Club called the "Eureka," in which he was joined by two or three other boys who have also become famous in the Biological sciences. From the academy he proceeded to the University, in Edinburgh, and in 1878 gained the Gold Medal in Wyville Thomson's Class for Comparative Anatomy. After taking his degree in 1879, an event occurred which to a large extent determined the principal line of research in which his name became recognised as the chief living authority. He was offered the large and valuable collection of Tunicata made by the *Challenger* Expedition, and set to work on his task with such characteristic energy that he was able to publish a preliminary report on the Collection in the 'Proceedings of the Royal Society of Edinburgh' in the year 1880. The full reports on this Collection were published in the Series a few years later—Part I, on the Simple Ascidians in 1882; Part II, on the Compound Ascidians, in 1886; and Part III, on the Thaliacea, in 1888.

In the winter of 1879 he was awarded the Baxter Natural Science Scholarship, and succeeded Mr. G. Leslie as Secretary of the *Challenger* Expedition Commission. The year 1880 was spent in assisting Wyville Thomson in the sorting and disposal of the vast amount of material collected by the naturalists of the *Challenger* Expedition, and no doubt the experience gained in this work over a wide range of the families of marine organisms was of great value to him in his career, and stimulated the ready sympathy and understanding which he always showed for the work of fellow zoologists in other branches of the subject. But he was soon called to assist in the teaching work of the University, and in the winter of 1881 was appointed Demonstrator of Zoology, a post which he held until November, 1881, when he was made the first Derby Professor of Natural History in the University College, Liverpool.

During the tenure of his demonstratorship in Edinburgh he organised and led dredging expeditions in tug-boats from Leith, some of the results of which were published in a series of papers on "The Fauna of the Firth of Forth" in the 'Proceedings of the Royal Physical Society of Edinburgh' in 1881.

\* The writer is much indebted to Miss Catherine Herdman and Prof. James Johnstone for assistance in preparing this obituary notice.



W. A. Herdman





His appointment to the Chair of Natural History in Liverpool gave him the opportunity, for which no doubt his keen and active spirit was longing, to initiate and carry through, in spite of many difficulties and disappointments, a great movement for the co-ordination of the interests of those engaged in purely scientific investigation and of those engaged in the fishing industry.

He saw with scientific acumen that the first important steps to take to bring about such a result were to make a thorough investigation of the fauna and flora of the seas of the district, apart from questions of their economic value, and to this end he gathered round him a body of enthusiastic naturalists, who spent what time they could spare from their various avocations in life in collecting, naming, and noting the distribution of the marine animals and plants of the Irish Sea. A small station was established on Puffin Island, off the North Coast of Wales, in 1887, and there he and his friends assembled in the vacations and carried out their operations of shore collecting, tow-netting and dredging.

The station on Puffin Island, however, was not only very difficult of access, and in many ways inconvenient, but it did not provide for his investigators the richness and variety of fauna and flora which Herdman required to carry out his schemes. The station was consequently removed to Port Erin, in the Isle of Man, in 1892, and there it remains—enlarged and rebuilt as the work was extended—as one of the great marine laboratories of the country, the centre of important scientific investigations of the sea, of practical education of succeeding generations of students of biology, and a worthy memorial of its founder.

The work of the station was continued in its new quarters with ever-increasing activity and usefulness. Numerous papers appeared in the 'Transactions of the Liverpool Biological Society' and elsewhere on the Fauna of the Irish Sea, and many important contributions to our knowledge of the anatomy and distribution of Tunicata and other marine organisms were made by Herdman himself. But the need was felt for a more detailed knowledge than we possessed of the anatomy and development of some of the commoner forms of animal and vegetable life, as a guide to the morphology of the groups they represent, and consequently it was decided to publish a series of memoirs by recognised authorities to meet this daily requirement of the student of biology.

The first of these memoirs, published in 1899, on *Ascidia*, was written by Herdman himself, and was followed in rapid succession by memoirs by various competent authorities on the cockle, the sea-urchin, and many other common animals and plants of our sea coasts. These memoirs, being sold at the low price of from 1s. 6d. to 4s. each, according to the size and cost of illustrations, have proved to be of great value to students interested in biology.

His connection with the fishery industry began in 1891, when he was invited by the Lancashire Sea-fisheries Committee to give a course of lectures in

Liverpool, specially adapted for fishermen, and in the following year he began to organise a small fisheries laboratory and to direct the observational work of the Committee. At the close of the same year the first of a series of annual reports appeared, on the investigations carried on in connection with the Lancashire Sea-fisheries Committee, which he edited until the time when he resigned the chair of Oceanography in the University. The work of the Committee increased rapidly under his energetic guidance, and in 1897 a laboratory was established at Piel, near Barrow, on the coast of Lancashire, which was intended at first to be used mainly for fish-hatchery experiments, but subsequently extended its activities to other and more generally scientific investigations.

The energy and skill with which Herdman had carried on his investigations on marine biology in the Irish Sea, and had brought successfully into contact the purely scientific work of himself and his associates and the practical economics of the fishing industry, led to his appointment by the Colonial Office as Director of a commission to investigate and report upon the Pearl Fisheries of Ceylon. He left Liverpool in the autumn of 1901, and after three months of strenuous work on board the S.S. *Lady Havelock*, dredging and collecting in the Cingalese waters, he returned to England and prepared his report. The valuable collections he made on this cruise were sorted out and distributed to various naturalists, and the results of this very important contribution to our knowledge of the fauna of these tropical seas were published in five handsome volumes by the Royal Society.

On his return from Ceylon he again entered vigorously into the marine investigations at Port Erin and at Piel. In association with several colleagues he carried on for several years an intensive qualitative and quantitative study of the marine plankton, which led to some results of far-reaching interest and importance. A summary of these results was published in 1922, in the fifth volume of his 'Spolia Runiana' and in the 'Journal of the Linnean Society—Botany,' vol. 46, No. 306.

The list of his scientific papers on various branches of zoology during these busy years is a very long one, and shows evidence of his extraordinary industry and wide interests and sympathies. All that can be said here is that he never lost his interest in the group of Tunicata, with which he began to make his mark as a zoologist, and that he contributed during his life many valuable additions to our knowledge of the systematics and morphology of these important, although degenerate, organisms.

The year 1905 was marked by an important event in his career, the handsome and commodious new Zoological Laboratories being declared open by Lord Derby, in the presence of a large and distinguished audience. On this occasion there was a fine display of the spoils of the Irish Sea, collected by the Liverpool School of Zoology, and many of those present, including the representatives of

the Board of Agriculture and Fisheries, were much impressed by the evidence of the practical results obtained by the work done in connection with the Lancashire Sea-fisheries Committee.

When the War broke out in 1914 and work on the seas became difficult and dangerous, he devoted a great deal of his time to war work in connection with the Royal Society, of which he was at the time Foreign Secretary, and he edited the "Reports of the Grain Pests (War) Committee." The war brought also to him a very great loss and a pitiful grief. His only son, a promising lad of 21 years, was killed in the Battle of the Somme in July, 1916. In his memory Professor and Mrs. Herdman founded and endowed the George Herdman Professorship of Geology in the University of Liverpool.

Herdman and his wife had an intense love of the sea and were frequently together on the steam yachts *Runa* and *Ladybird*, carrying on investigations into the plankton and coming into touch with many other scientific problems associated with them. It was felt, however, to be desirable that all these problems should be brought together into an organised unit, and consequently they founded and endowed the first Chair in this country of the Science of Oceanography. Herdman retired from the Professorship of Natural History in 1919 and occupied the new Chair of Oceanography for one year, being succeeded in 1920 by Prof. James Johnstone.

Retirement, however, could not mean for a man of his spirit a time of leisure. His interest in his University never flagged, and for a period he undertook the arduous duties of the Vice-Chancellorship. He brought together in a short but most valuable paper the general results of 15 years' investigations on the plankton of the Irish Sea, and he published the only large illustrated book we have from his pen—'The Founders of Oceanography and Their Work'—a book which apart from its interest and charm is, and must remain, a standard work of reference on the history of marine investigations.

He received the honour of knighthood in 1922, but at the close of that year suffered another serious bereavement by the sudden death of Lady Herdman, who had for 30 years been a constant helpmate and sympathiser in his various activities. In her memory he gave to the University of Liverpool the sum of £20,000 for the building of the Geological Laboratory.

Herdman was twice married, first to Sarah Wyse Douglas, of Edinburgh, who died in 1886, leaving two daughters, who married respectively Prof. H. E. Roaf and Dr. Montague Phillips, and secondly to Jane Brandreth Holt, of Liverpool, by whom he had two children, the son George who was killed in the war, and a daughter Catherine who has inherited her father's love of zoological science.

In the course of his strenuous life, Herdman received many well-deserved honours. He was elected a Fellow of the Royal Society in 1892, when he was 34 years of age. He served on the Council of the Society as an ordinary member from 1898–1900 and as Foreign Secretary from 1916–1920. For many years he

was one of the General Secretaries of the British Association and was made President for the Cardiff Meeting in 1920. He was made an LL D. of Edinburgh University and an honorary D.Sc. of six other universities. He was made a C.B.E. in 1920 and was knighted in 1922.

On July 21, 1924, when on a visit to London, he was seized with a sudden heart attack in the street, and died before medical assistance could be obtained.

S. J. H.

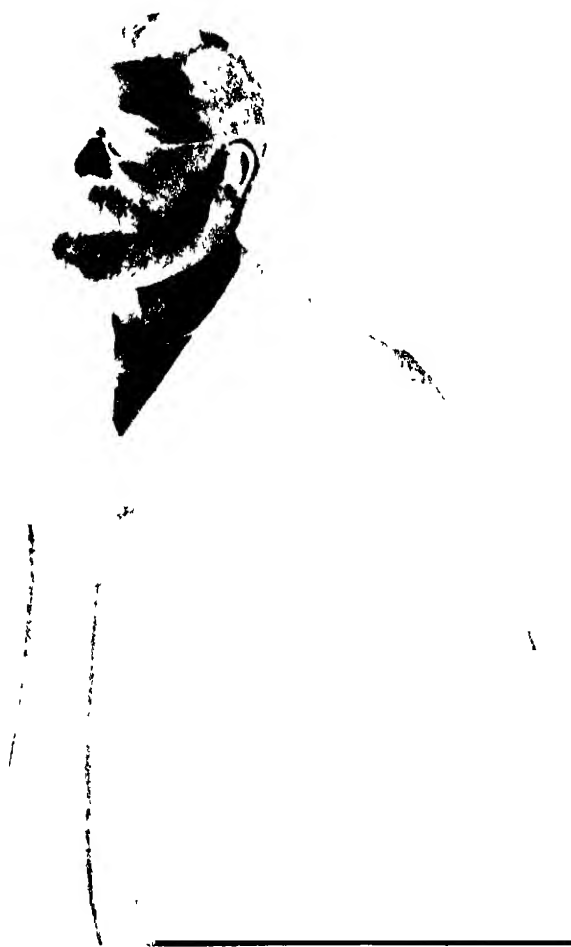
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ROBERT KIDSTON—1852-1924.

ROBERT KIDSTON was born at Bishopton House, Renfrewshire, on June 29, 1852, but from early boyhood his life was spent in the ancient town of Stirling. He died at Galfach Goch in Wales, after a very brief illness, on July 13, 1924.

As a boy his taste had been for the sea, but, after his education at Stirling High School, he entered the service of the Glasgow Savings Bank. His business career soon closed with the failure of the bank in 1878, and his private means enabled him to give his time wholly to his scientific interests. He attended the class of botany at the University of Edinburgh in the summers of 1879 and 1880, obtaining First Class Certificates, and a medal in Practical Botany. He did not take a full curriculum or graduate. At Stirling he lived at first with his unmarried sisters, of whom he took the greatest care throughout his life. In 1898, on his marriage with Agnes, younger daughter of the late Major Oliphant, of Over Kinedar, Fife, he removed to an adjoining house with an ample garden. To this house he added a wing including accommodation for his scientific work, and it was his home with his devoted wife and two daughters for the rest of his busy and happy life.

Fully occupied with his studies, he yet took part in the life of the town, and his judgment was valued in political and church affairs. He was a magistrate for Stirlingshire, and during the war was selected as chairman of the tribunal, an expression of the general sense of his conscientiousness and fairness. A more individual contribution to the needs of that time was the part Mrs. Kidston and he took in collecting and preparing bog-moss for use in surgical dressings. This was begun on a small scale, but Kidston's practical sense led him to organise a voluntary collecting camp, to provide the right kind of moss in quantity. In this task he worked hard himself and inspired enthusiasm in all his helpers.



*W. H. H. H.*



Always interested in living plants, he was a keen gardener and few days passed without his visiting his rock-garden and his collection of Saxifrages. Other recreations, taken sparingly but enthusiastically, were curling and fishing, while sketching and photography added to the interest of his travels. It was his nature to do everything he undertook as well as it could be done, and this held in daily life as well as in science. It was evident to all who came in contact with him that this kindly, humorous, shrewd Scot was no ordinary man. But it may be doubted if any of his fellow-townsmen realised that the quiet private student, who passed his whole life among them, had become the unquestioned authority on plants of the coal-measures and still earlier periods, and that his advice was sought from all over the world.

Duties in connection with learned societies and academic honours came to him in due course. He was made an Honorary Graduate of the Universities of Glasgow and of Manchester. Early in his career he became a member of the Royal Physical Society of Edinburgh (1878) and a Fellow of the Royal Society of Edinburgh (1886), and took an active part and held important offices in both. He was elected a Fellow of the Royal Society in 1902. He was also a Fellow of the Geological Society of London and was an Honorary Member of a number of foreign learned societies. The Royal Society of Edinburgh awarded him its Neill prize in 1890, and he received the Murchison Medal in 1916. In acknowledging the medal to the Geological Society, he mentioned that the balance of the Murchison fund, awarded him in 1887, had been devoted to the purchase of working books and the "hope that the books will eventually be placed where they will be of help to others." He carried out this intention by bequeathing his valuable library on fossil plants to the Botanical Department of the University of Glasgow.

While these are some of the main outward events of his career, its essential interest lies in the history of his scientific development. Little can now be gathered as to the first beginnings of this. He must have been a collector and interested in Natural History throughout his early youth, and we know that he attended lectures when in the bank at Glasgow. Some of these were by Williamson, and this first directed his attention to fossil plants as a promising field of work. Notes of scientific excursions, which he kept from 1879, show that he began by studying the quarries and pits near Stirling and naming the fossil plants, not merely from the coal-tips but down in the mines. His serious study of palæozoic plants was thus evidently determined on early, but he continued to take an active interest in other branches of Natural History. Many of the expeditions he records were to collect diatoms, and another series of excursions was connected with the study of the flora of Stirlingshire. These led him to all parts of the county along with his friend, Colonel Stirling. Ten short papers on this subject, in the 'Transactions of the Stirling Natural



History Society,' between 1891 and 1899, are his only published work on subjects other than palæobotany.

His long and cordial relations with the Geological Survey began early and were a source of mutual advantage. Their foundation was laid during his time in Edinburgh, and, from about 1880 onwards, all palæozoic plants collected by the Survey were submitted to him and he acted as voluntary plant-palæontologist. He did not merely determine specimens sent to him, but, whenever this seemed desirable, joined the members of the Survey, among whom he had many friends, in their field-work. His private collection of hand-specimens of British palæozoic plants steadily grew, every specimen being labelled with scrupulous exactness as to its locality and horizon. He spared no pains in adding to the collection, which became one of the finest in the world. This was in no selfish spirit, for it was the embodiment of the work he was doing and was always destined for future public use. This intention also he carried out, by bequeathing the complete collection, with all the notes and catalogues relating to it, to the Geological Survey. This important addition to the national collections will be of the greatest value in the study of British coalfields.

The record of Dr. Kidston's scientific work is in the long series of papers which he published from 1881 to 1924. Only the outstanding results of this work can be touched on here. It is interesting to note that his earliest paper dealt with the anatomical structure of a petrified plant. But it was not until after 1904 that this side of his work assumed great importance and it will be treated as a whole below. For a time he concentrated on the study of fossil plants preserved as incrustations, and almost all his earlier publications deal with these. Beginning with his acute study of the specimens he had himself collected, his knowledge grew so rapidly that in 1886 he was asked to prepare the Catalogue of the Palæozoic Plants in the British Museum. Valuable as this was, his own work soon rendered it out of date as a list of the British Carboniferous plants. The immense advance in our detailed knowledge of these during Kidston's life-time can be gauged by comparing the Catalogue with the great Memoir for the Geological Survey, upon which he was engaged at the time of his death, and which would have summed up his unrivalled knowledge of the subject. So largely does the advance depend on Kidston's own labours that if, as is to be hoped, it is found possible to complete the Memoir, it will have to be on the basis of his publications and collection.

To survey the numerous papers which formed the foundation for this work is impossible here. They include descriptions of interesting specimens, annotated lists of collections submitted to him for examination, full accounts of the floras of various coal-fields and monographs of particular genera. While mainly concerned with Britain, his help was sought by other countries and two important pieces of work had their origin in the investigation of

Belgian and Dutch coal-fields. As critical accounts of the floras of particular localities, and as contributions to difficult problems in the interpretation of fossil plants, they are all of permanent value. The accuracy of their illustration, at first by careful drawings and later by photographs of such perfection that they almost replace the specimens for purposes of study, enhances the value of the clear and concise descriptions. The general comments are as important and authoritative as they are brief.

Throughout these systematic or floristic papers there is a wealth of accurate description of fine specimens, which, in adding to our knowledge of the characters of extinct plants, have important bearings on general morphological botany. The reproductive organs of the simpler types of *Sphenophyllum*, the cones of *Sigillaria*, and the fructifications of a number of Ferns and Fern-like plants may be referred to as illustrations of this. Other papers dealt specially with morphological problems, which were always defined and illuminated by the facts that Kidston was able to demonstrate. A number of the most important of these contributions bear on the early history of Seed-plants. About the beginning of the century the suspicion had grown that many of the Fern-like plants of Carboniferous times were Gymnosperms, and this had been expressed by the recognition of a group of Cycadofilices. The evidence came from the anatomy of the vegetative organs, but in 1903 Oliver and Scott established a probability so strong as to be practically a demonstration, that the complex seed *Iagenostoma* was borne on the foliage of *Lyginopteris*. This brilliant discovery, originally due, as Dr. Scott has pointed out, to Prof. F. W. Oliver, was based on the comparative study of petrified remains in the coal-balls.

In the interval between these investigators' preliminary statement and full paper, Kidston demonstrated the attachment of a large seed, enclosed in a cupule, to the foliage of *Neuropteris heterophylla*, another Carboniferous "Fern," and was also able to show that a specimen he had described many years before represented the pollen-bearing organs of the same plant. This direct evidence from the incrustations of *Neuropteris* thus supplemented the more indirect evidence which Scott and Oliver had obtained for *Lyginopteris*, and the class of *Pteridosperms* was securely established. The paper containing Kidston's contribution to this problem was published in the 'Philosophical Transactions' and was followed two years later by another, "On the Microsporangia of the Pteridospermeæ, with Remarks on their Relationship to Existing Groups." The microsporangia of *Lyginopteris* were here identified and fully described, thus completing the work of Oliver and Scott on this plant. The general discussion is especially important, because Kidston's more cautious attitude as to the derivation of the Seed-plants from Ferns has been justified by the further progress of knowledge. A brief, final summary of his opinions on the whole problem, to which he made other contributions, was given in the introduction

to the Memoir on "Fossil Plants of the Carboniferous Rocks of Great Britain."

It has already been mentioned that Kidston's earliest paper dealt with the anatomical structure of a petrified plant. This was a stem of *Lepidodendron selaginoides*. Faithful as it is in its description and illustration, it suggests that the botanical teaching of the time at Edinburgh was less adequate as a preparation for this side of palæobotany than for systematic work. But the paper is of importance, for it gives an account of the periderm and the position of its meristem that is both correct and original. With the sound instinct that guided him, Kidston proceeded to devote himself for many years to the description of incrustations, where he felt on sure ground. But during this period he was making a large and important collection of petrified plants and studying them carefully. Though for a time he did not publish the results of his observations, he materially advanced scientific knowledge by the assistance which he rendered, with characteristic generosity, to other investigators. Dr. Scott has told me how he made Kidston's acquaintance when reading a paper at the British Association in 1896. In the discussion that followed, Kidston said that he had a cone in his collection that might shed light on the problem. The result was that Dr. Scott at once paid a visit to Stirling, examined the sections and was taken to Pettycur to see the locality. The preparations and the block from which they had been cut were placed at his disposal and resulted in his important paper on the complex cone of *Chevrostrobus*. At that time Kidston was still living with his sisters, and Dr. Scott recalls the keen interest they took in the work. Then, and on subsequent visits to Stirling, he saw many of the slides in the collection, and this supplied him with much of the material for two other papers, published in 1902 and 1924, on the stems of Carboniferous Gymnosperms. These unselfish scientific relations are only one example of the way in which Kidston drew freely on his collection to further the work of others.

The bequest of this valuable collection of more than 3,000 slides, including hundreds of figured specimens, to the Botanical Department of the University of Glasgow has peculiar appropriateness. For Kidston's return to active publication on the internal structure of fossil plants was intimately associated with the close relations that were established in 1904 between him and the Glasgow Department, and, in particular, to the collaboration between him and Gwynne-Vaughan. Gwynne-Vaughan and I first saw and heard Kidston in 1899, when he gave his address on Carboniferous Lycopods and Sphenophylls to the Glasgow Natural History Society, but we only came to know him at the Cambridge Meeting of the British Association in 1904. The friendship developed rapidly in the following winter. In my own case, and I am sure in Gwynne-Vaughan's also, the most important and valued influence in our mature scientific lives was the privilege of working with Kidston.

During the winter of 1904 and in subsequent years, Kidston came, about once

a week, to the Glasgow laboratory to study existing plants, on which he felt the need of further knowledge for comparison with fossils. He was as much at home in the assistants' room as in Prof. Bower's, and his genial presence as a luncheon guest was keenly in demand by all the members of the Department. At this period, also, there were visits of Gwynne-Vaughan and myself to Stirling to be shown fossil plants in the collections. I vividly recall our first visit; met, as always, by Kidston standing on the platform, smoking his characteristic white pipe; tea in the drawing-room, and our first acquaintance with the family; and the first evening in the study that was to become one of the best known and best loved places to both of us.

That study was like no other room that I have known. With its collections of palæozoic plants and fossil slides, its apparatus and its working collections of books and pamphlets, it was museum, laboratory, library and study in one. It was a complex tool fashioned for Kidston's work, or rather it had grown as the natural extension of his personality. Everything he needed was there in its proper place, and he could lay his hand on any specimen, unworked material or apparatus he required. The spacious well-lit upper room had a feeling of Spartan comfort, and in its isolation from official or academic science, was an ideal place for getting work done for its own sake. But, as was shown by the photographs of his scientific friends, including Nathorst and Zeiller and all the main students of fossil botany in Europe, there was no scientific isolation. Kidston would have been a great investigator even if he had held an official post, but the peculiar character of his work is, I think, to be associated with the individuality of its conditions, and these were expressed in the study at Stirling.

There was a rare and instinctive sympathy between Gwynne-Vaughan and Kidston. Joint work was soon in progress, foreshadowed by an acknowledgment of help in the important paper on the internal structure of *Sigillaria elegans*. This, and a number of other pieces of anatomical work by Kidston alone, show how his association with one who was skilled in modern work on plant anatomy and at home with the current theories, had raised the inhibition that had rested on his publishing the observations he had made on the structural part of his subject.

The series of five joint papers by Kidston and Gwynne-Vaughan on the fossil Osmundaceæ can be mentioned briefly because of the unity of their subject. The co-operation of the palæontologist and the plant anatomist, both masters in their craft, resulted in the production of what is recognised as a botanical classic. The anatomy of the stem and petiole was fully described and illustrated in a number of specimens that form one of the most remarkable evolutionary series that is known in plants. The line of the Royal Ferns of to-day was traced back, on convincing anatomical evidence, to Permian-carboniferous times, and an earlier community of origin with the Botryopteridæ was made highly probable. Two other joint investigations were published, one

on *Tempskya* and the second on *Stenomyelon*. The latter was intended to be followed by an account of other Lower Carboniferous plants from Berwickshire.

Gwynne-Vaughan's death in 1915 was not only a great loss to botany, but a very special blow to Kidston. Shortly afterwards I was on a visit to Stirling and we discussed the possibility of continuing the joint work on which they had been engaged. But, on my being shown the first sections of the Rhynie Chert, we decided to defer the further description of the Lower Carboniferous plants and to concentrate on those of the Old Red Sandstone.

The importance of the plants preserved in the Rhynie Chert, believed to be of Middle Old Red Sandstone age, led to the material being investigated as completely as possible. The two papers at first projected, expanded to five, and the continued aid of the Carnegie Trust enabled the Edinburgh Royal Society to illustrate these fully. The series dealt with the four vascular plants belonging to the new genera *Rhynia*, *Hornea* and *Asteroxylon*, and with the remains of lower plants which accompanied them in the silicified peat. Although the remains in this were fragmentary, the pieces were so numerous, and represented all parts so completely, that it was possible to reconstruct the external appearance of all the plants. Their morphology was so simple and peculiar that a new class of Pteridophyta, the Psilophytales, had to be founded upon them. Since their internal structure was also preserved in wonderful perfection, the Rhynie plants became known more fully than any other members of the Early Devonian flora, and have stimulated a renewed interest in this in more than one country.

Kidston's interest in the flora of the Old Red Sandstone was of long standing. He had collected in the lower rocks of Forfarshire and Perthshire, and in 1893 had visited Wick and Thurso to examine the Middle Old Red Sandstone plants. His early publications on these, though few compared with his work on the more intelligible Carboniferous flora, are all definite advances made wherever this seemed possible. *Archæopteris*, *Ptilophyton*, *Arthrostigma* and *Cryptoxylon* were thus described, while some short notes dealt critically with the flora as a whole. With the fuller understanding afforded by the Rhynie plants the project of a systematic account of the Devonian flora of Britain was contemplated, and the joint papers that followed the Rhynie series were in preparation for this. They included the first fully illustrated account of *Palæopitys Milleri*, the woody stem discovered by Hugh Miller in 1841, and, at the other extreme of the plant kingdom, a study of the peculiar Alga, *Pachytheca*.

The full account of the flora had, however reluctantly, to be deferred, when in the latter part of 1922 the preparation for the Geological Survey of the Memoir on "Fossil Plants of the Carboniferous Rocks of Great Britain" took precedence of everything else, and absorbed almost all Kidston's time and energies. He retained his keen interest in the work on the Old Red Sandstone plants, but kept the microscopical examination it involved for a relaxation in the

evenings. Though his inclination would have been to proceed with the relatively unknown flora, the opportunity for gathering together his life-work on Carboniferous plants was felt as a paramount duty.

Six parts of this great work, almost completing the Fern-like plants, were finished at the time of his death, and form the first volume. They constitute the most authoritative and full account of the plants of which they treat, both from the point of view of description and of their exact geological distribution. Had Kidston lived to complete the Memoir it would have summed up and made available for future workers the fruits of his long labours and unique experience. It was done at a pressure that proved too great even for his capacity for sustained work. The strain was increased by the fact that he could not be content with the store of knowledge he possessed or with the material in his collection. Such artificial finality was not his way. So he continued to add to his knowledge, and his last, short illness seized him while on a visit to South Wales to examine a large set of fossils. He was working as actively as ever almost to the end. Nothing could show more clearly what an irreparable loss to British palæobotany Kidston's death was, than this great unfinished work, which there is no one to complete as he would have done it.

Kidston's scientific publications fall naturally into four groups and on each subject he made important contributions. He worked up the British Carboniferous flora to a high degree of completeness and related it to the geological horizons. He made numerous discoveries on the morphology of Carboniferous plants, notably in connection with the reproductive organs of the Pteridosperms. He contributed to our knowledge of the anatomy of Permo-carboniferous plants, especially in the case of the Osmundaceæ. And lastly he made fundamental contributions to our knowledge of Old Red Sandstone plants.

The esteem in which his work was held throughout the world is expressed in a sentence from a letter which I received from Dr. M. Zalessky, himself a distinguished Russian investigator in all the fields in which Kidston worked. "*Il était le chef de travail paléobotanique dans votre pays, et ce soleil d'Ecosse éclairait par sa lumière lumineuse tous les paléobotanistes de l'Univers et les excitait aux recherches nouvelles.*"

Kidston was one of the great amateurs of science, working for the pure love of discovery. Perhaps it was because of this that he spared himself so little and accomplished so much that is of permanent value. Endowed with the combination of qualities required for the best work in his chosen subject, industry, accuracy and critical caution, his insight and imagination were always under the strict control of well-tested observations. While this explains the high quality and reliability of all his work, its quantity was only possible because of the sound organisation of his time and energies. It is striking how at every critical point in his life he saw "toward solid good what leads the nearest way," and his orderliness and method carried the same instinct into details.

In his work, and indeed in many other respects, Kidston remained young. He approached every question with real and vivid interest, held closely to the facts and drew his inspiration direct from them, distrusting merely speculative theories. His keenness in the discovery of something new, and in the piecing together of facts thus brought out, was a perpetual stimulus to all who came in contact with him at work. While he did not lightly change opinions he had formed, and required proof from the specimens for this, he was always ready to look for the evidence against his own views. He liked the most direct discussion in eliciting the truth of a matter, and he had a very keen sense of humour to which he gave full play in his work. This came out strongly in joint investigations, and the uncompromising arguments that took place in the study at Stirling and the healthy atmosphere of banter on the work that reigned there, were not only enjoyable but to the good of the final result. Kidston was the most modest of men, always ready to learn and especially encouraging to beginners at his subject. Indeed he responded to sincerity in every form, for he was himself absolutely genuine and big enough to be quite simple. And his keenness, sagacity and kindly humour were not for his work only, but for the whole of life. He was a wise counsellor and a delightful companion and inspired liking and love in all who knew him. For behind and above all was the charm of a personality which was even greater than his knowledge and insight.

W. H. L.

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*Henry Woodward.*

## HENRY WOODWARD—1832-1921.

**HENRY WOODWARD**, who was born at Norwich on November 24, 1832, and died at Bushey, Herts, on September 6, 1921, was the fifth son of Samuel Woodward, the geologist and antiquary. His father died when he was only five years old, and his early life was spent under difficult circumstances. He was educated at the Norwich Grammar School until 1846, when he went to reside with his brother, Dr. S. P. Woodward, who had been appointed Professor of Natural History in the Royal Agricultural College, Cirencester. Here he became an external student for three years, and spent his leisure in collecting fossils, shells, insects and plants in the Cotswold Hills. In 1849 he removed to London with his brother, who had been made an assistant in the Geological Department of the British Museum. In London he obtained some temporary occupation, but it scarcely sufficed for his needs, and in 1851 he returned to Norwich as clerk in Gurney's Bank. He remained in the bank for seven years, gaining useful business experience, until, in 1858, he realised his ambition and followed his brother as assistant in the Geological Department of the British Museum. He was eventually promoted to the Keepership in 1880, and retired under the superannuation rule in 1901.

As an officer of the British Museum Henry Woodward worked hard at his congenial duties as Keeper of the fossils, and soon acquired an extensive knowledge of certain groups, which he utilised in making original contributions to geology and palæontology. When he became Keeper in 1880 the collections were being removed from Bloomsbury to South Kensington, and he planned and supervised the rearrangement in the new building. He tried to make the public galleries more interesting and instructive by the addition of descriptive labels and diagrams, and he wrote new guide books illustrated with figures of important specimens. He also increased the value of the collections by planning and editing a series of more or less descriptive catalogues. His genial personality and helpful spirit attracted an increasing number of visitors, and as a result of his efforts the scope and extent of the collections enlarged with greater rapidity than at any previous time.

In 1864 Henry Woodward did good service to science by founding the 'Geological Magazine,' which he continued to edit until his death. For the first six months, in deference to the publishers, who insisted on the guidance of an older editor, he was associated with Prof. T. Rupert Jones, but for the rest of the time he was almost sole editor, with only a small number of advisers. Under his wise direction the magazine always welcomed and published serious work, whether orthodox or otherwise, and it afforded a unique medium for discussion. Besides many of his original papers Woodward contributed to

it several notes on new specimens exhibited in the British Museum. Among the latter may be specially mentioned his note on the skull of the mammoth from Ilford, Essex, in 1868, which proved that in the published figures of the Siberian mammoth in Petrograd the tusks were reversed.

Henry Woodward's chief contributions to science, however, were his descriptive memoirs and papers on fossil crustacea and other arthropods, on which he became a leading authority. In 1864 he exhibited to the British Association at Bath some restorations of the Devonian merostomata. and between 1866 and 1878 he contributed a monograph of this group to the publications of the Palæontographical Society. For this society he also wrote monographs of the Carboniferous Trilobites (1883-84) and the British Palæozoic Phyllopoda (Phyllocarida) (1888), the latter with Prof. Rupert Jones. His smaller papers related to numerous specimens of other groups, and in 1877 he summarised these to date in his Catalogue of British Fossil Crustacea, published by the British Museum. During more recent years he extended his researches to Carboniferous insects, myriapods, and arachnids, especially from the English coal measures. He wrote on Tertiary shells from Sumatra (1879), Palæozoic fossils from Beechey Island (1878), and a well-preserved cuttle-fish from the chalk of Mount Lebanon (1883). All his papers were well illustrated, many by his daughters.

Henry Woodward's numerous writings brought together useful materials for science, but rarely did more than state the facts and make a few obvious comparisons. Although admitting the general truth of the doctrine of evolution, he left to others its application to palæontological research. At the same time he encouraged younger workers to adopt the new methods, and he was ever ready to help them with his experience and judicious criticism.

Henry Woodward also took a very active part in the work of several of the London societies. After long service on the Council of the Geological Society, he was elected President in 1894, and received the Wollaston Medal in 1906. He was an early member of the Geologists' Association, became President in 1873, and from 1875 onwards was an honorary member. He was long a member of Council of the Palæontographical Society, and in 1895 he succeeded Prof. Huxley as its President. He was President of Section C of the British Association at Manchester in 1887. He was the first President of the Malacological Society in 1893, and presided over the Royal Microscopical Society in 1902-4. For many years he was also an active Vice-President of the Zoological Society. He was elected a Fellow of the Royal Society in 1873, and received the honorary degree of LL.D. from St. Andrews in 1878.

In 1857 Henry Woodward married Miss Ellen Sophia Page, of Norwich, who took a keen interest in his scientific work, and gave him much help until her death in 1913. Two sons, who died prematurely, made a promising start

in science, and one of his five daughters, Miss Gertrude M. Woodward, is well known for her beautiful illustrations of fossils and zoological specimens in the writings of many authors. The hospitable receptions of Dr. Woodward and his family, when he was Keeper of Geology, will long be remembered by those who had the privilege of his friendship. His was a cheery personality which it was good to have known.

A. S. W.

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#### EDWARD EMANUEL KLEIN—1844—1925.

EDWARD EMANUEL KLEIN, who died on February 9, 1925, was a figure little known to the present generation, for he had lived in complete retirement for more than 10 years, and the principal work of his life was accomplished a quarter of a century ago. Yet at one time he occupied a prominent position in the world of medicine and public health, and played a great part in the early development of bacteriology in this country. His original name was Emanuel Klein, and the additional name of Edward was assumed long after he became resident in this country. He was born at Essek, the capital of Slavonia on October 31, 1844, and was thus of Austro-Hungarian nationality. His father is said to have been a tanner of Russian leather; he died during Klein's boyhood. Klein received a good education, becoming familiar with the classics and speaking German, Hungarian and French. At a comparatively early age he was able to contribute by teaching to the support of his mother and two sisters. With the aid of scholarships he proceeded to the University of Vienna, studying medicine in what was already a school of high repute and in due course taking the degree of M.D. His interests were with the scientific aspects of medicine rather than with its practice, and he became attached to Stricker's laboratory, where his abilities raised him to the rank of "Privat-dozent." Here Klein acquired the remarkable technical ability in histological work which was later to stand him in such good stead.

It happened that, in 1869, the New Sydenham Society determined to publish an English translation of Stricker's 'Manual of Human and Comparative Histology,' a work to which Klein himself had contributed important sections, namely, those on the thymus gland, the external generative organs, and the serous membranes, and, in part, on the intestinal canal and the eye. Stricker, requiring someone to proceed to England to negotiate the details of the

translation, not unnaturally selected Klein for the purpose, furnishing him with introductions to some of the leading scientific men of this country, including Huxley, Burdon-Sanderson, Simon and Henry Power, the last-named of whom was to undertake the translation. Although he spoke no English at that time, the handsome presence and pleasing address of the young foreigner created a favourable impression on those with whom he came in contact. He accomplished his task with success, and returned to Vienna to resume his histological and embryological work in Stricker's laboratory.

In order to understand the circumstances which led to Klein's return to this country it is necessary to recall certain facts in the history of sanitation and public health in England. The great apostle of the reform of the public health services was Sir John Simon, who had been Medical Officer to the Privy Council, and was now to become also Chief Medical Officer to the Local Government Board. The nature of contagion—a problem to which the work of Pasteur had provided the key—was necessarily of deep interest to those concerned in the prevention of infective disease, and Simon had induced Burdon-Sanderson to take up this subject of study. Sanderson's first report on 'The Intimate Pathology of Contagion' appeared in 1870, and the work appeared to Simon of such importance that, in the same year, Parliament sanctioned a grant of £2,000 a year, to be administered by the Local Government Board in furtherance of similar studies. About this time, too, the Brown Institution for the Study of Animal Diseases was founded at Wandsworth. This was a convenient place for the purpose of work of this kind.

With the new financial grant it became possible to secure assistance for Sanderson, and as a resident director for the Brown Institution was needed, Simon and Sanderson bethought themselves of the young Slav who had made so favourable an impression on them in his recent visit. On Stricker's recommendation Klein's services were obtained in the spring of 1871. Doubtless one of the reasons which led him to accept the invitation was the high cost of living in Austria. He had seen the Prussians at the gates of Vienna, and the country was reduced to great financial straits.

By training, Klein was at that time a pure histologist, and one of brilliant promise. There is little doubt that, had he remained in Vienna, he might have left his mark as one of the foremost histologists in Europe. It was, indeed, as a microscopist that his services were demanded, for it must be remembered that, at that period, the microscopic method was the only one which appeared feasible for the study of infection. Bacteriology, as we now understand it, with its accurate cultural and experimental methods, was not founded by the genius of Koch till several years later.

Although Klein was destined to become a bacteriologist, and although it was in that capacity that he later became chiefly known, it will be convenient to consider first his position as a histologist, since for some years after his

arrival in this country his work was mainly histological and he never lost his interest in the subject. During the first few years at the Brown Institution he carried out important researches on the histology of the lymphatic system, devoting himself chiefly to the serous cavities and to the lung. This work was primarily in relation to the spread of tuberculosis, but it developed into a purely histological monograph. Klein had at his command the methods introduced by v. Recklinghausen, and these, with many others, he employed with such good effect that many gaps in knowledge were filled. This work was published, in 1873 and 1875, in two small volumes entitled 'The Anatomy of the Lymphatic System,' and it did much to establish Klein's reputation as a histologist in this country. At the same time he wrote the histological part of the 'Handbook for the Physiological Laboratory,' by Burdon-Sanderson, Foster, Brunton and Klein—a work which for many years was indispensable to physiological students.

In 1873, Klein was appointed lecturer on Histology at St. Bartholomew's Hospital, and thus inaugurated a connection with that institution which was to last some forty years. He had by now mastered sufficient English to lecture in that tongue, though his pronunciation was still defective. The medical student of those days was a more ribald person than now, but Klein's courage, good nature and profound knowledge of his subject won their way, and he became a popular and successful teacher. At first he shared the teaching of physiology with Marrant Baker, but from 1884 to 1900 he was the sole lecturer: from the latter year onwards he had Dr. J. S. Edkins as coadjutor, and he finally retired from the post in 1902, becoming Lecturer on Advanced Bacteriology.

Klein wrote two further books on histology, both of outstanding merit. In 1879, appeared the 'Atlas of Histology,' produced in collaboration with Noble Smith. It is not too much to say that this is one of the most beautifully illustrated works ever published on the subject, and for this Klein's admirable preparations deserve as much credit as Noble Smith's artistic skill in reproducing them. Ten years after this he wrote a small text-book in collaboration with Edkins, called the "Elements of Histology"—a compact and authoritative student's manual which enjoyed great success and was translated into other languages.

In spite of his eminence as a histologist, the force of circumstances drove Klein to become a bacteriologist, and before passing any verdict on his success in this sphere, it is only just to recall the state of knowledge at the time he approached the subject. Bacteriology, as a science, did not exist: here and there in Europe a few workers were stumbling towards the light, but of teachers and even of literature there were at that time practically none. In this country Lister alone was working, up in Scotland, at the early stages of his epoch-making development of antiseptic surgery. At the Brown Institution,

Sanderson and Klein had to rely upon such microscopic observations and crude experiments as they could themselves devise. Klein was, indeed, entirely a self-taught bacteriologist.

He was, however, a diligent student of Continental literature and was eager to adopt any new technical methods which were published: his marvellous manual dexterity enabled him to turn these to good account. His earliest recorded cultural experiments were in 1878, on the virus of "pneumo-enteritis" in the pig; he succeeded in cultivating a virus in hanging-drops of aqueous humour, and with the third and fourth generations he claimed to reproduce the disease in animals. When Koch introduced the use of solid culture media, Klein soon became an expert in their employment.

His industry was prodigious, and almost every volume of the Reports of the Medical Officer to the Local Government Board from 1874 onwards contains one or more papers from his pen, on the most varied subjects relating to infective disease. He contributed papers to the Royal Society, of which he was elected a Fellow in 1875, serving on the Council at a later date (1888-90). It would serve no good purpose to enumerate all the papers which he wrote: some were still histological, for example, those on the morbid histology of typhoid fever and of scarlatina—work of permanent value to pathology. It was in the nature of things that his work in bacteriology was of a pioneer character, and though much of it has left little impress upon the science, many of his observations have stood the test of time. He early recognised the significance of streptococci in relation to scarlet fever (1886-7), and he discovered the causal agent of fowl enteritis. Later, he did important work on the pollution of water, and at the close of his active life, when he became associated with the Fishmongers' Company, he worked out and confirmed on a large scale the standards of sewage pollution in shellfish which are still in use.

With all this he made many mistakes. In 1885, after his visit to India to study cholera, he found himself in opposition to Koch as regards the causal part played in that disease by the latter's "comma bacillus": yet he later had to admit that Koch was in the right. His observations in 1887 on the relation of the "Hendon disease" in cows to human scarlet fever, and, a few years later, his somewhat similar observations in regard to diphtheria in cows, have not been confirmed, and are now regarded as erroneous, important as they appeared at the time. The vast importance of adequate control observations was hardly realised at the time Klein did most of his bacteriological work, and it is hardly fair to judge it by the standards of the present day. But it has to be admitted that in spite of the opportunities which lay before the early workers in bacteriology, he failed to enrich the science by any one discovery of first-class importance. Nevertheless, such a judgment would greatly underestimate the part which Klein played in the development of the new science; indeed, it might truthfully be said that he was the father of bacteriology in

this country. Lister was devoting himself to one specialised branch of the subject, and for a decade Klein was the only general bacteriologist in England and for nearly three decades the most prominent exponent of that science. His numerous writings served to draw the attention of the medical and lay public to the importance of bacteriology, but it was above all through his pupils that he exercised the most lasting influence on the development of the subject.

As soon as his work became known, some of the more progressive men in the medical profession began to resort to the Brown Institution, to receive such instruction in bacteriology as Klein was able to supply. Later, when he left that Institution about 1890, he established laboratories in a house in Great Russell Street, and here he gave more formal classes to a band of learners. A year or two later laboratory accommodation was found for him at St. Bartholomew's Hospital, and for many years he had pupils working under him. Thus he trained a number of workers whose names have since become well known in the world of bacteriology, and this at a time when it was difficult or impossible to obtain such instruction anywhere else in this country. All his pupils, amongst whom were Frederick Treves, Ronald Ross, Alexander Houston, Mervyn Gordon, the present writer and many others, will bear witness to the excellence of the technical instruction which they received, and to the help and encouragement which Klein was always ready to bestow; it may fairly be said that the impetus which he gave to the new study in England far outweighed any mistakes he may have made in his own research work. He wrote one small text-book on bacteriology, first published in 1884, and entitled '*Micro-organisms and Disease*'—a work which presented a very adequate survey of the existing field of knowledge at that period. It may be feared that, when the present generation of bacteriologists has passed away, Klein's services in the development of the subject will be largely forgotten, and in any event he will probably be remembered chiefly for his eminence as a histologist.

Klein became a naturalised Englishman, and in 1877 he married Mrs. Sophia Amelia Mawley, by whom he had a son and two daughters. His family life was a happy one: he was devoted to his wife and children, and his pleasant manners and kindly disposition rendered it a privilege to be admitted to his home circle. At one time he had been bitterly attacked on account of his evidence before the Royal Commission on Vivisection, but in truth he was humane and fond of animals. He was an ardent chess-player, resorting constantly to the British Chess Club, and he was sufficiently good to meet the most distinguished players under only a small handicap. He was also very fond of music. On his retirement from active work, he lived for a time at Chislehurst, and later at Earl's Court; the last year of his life was spent at Hove, in Sussex, and here at 80 years of age he died.





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